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Biotechnology and Nanotechnology Risk Assessment: Minding and Managing the Potential Threats around Us

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Biotechnology and Nanotechnology Risk Assessment: Minding and Managing the Potential Threats around Us

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Foreword

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Before agreeing to publish a book, the proposed table of contents is reviewed for appropriate and comprehensive coverage and for interest to the audience. Some papers may be excluded to better focus the book; others may be added to provide comprehensiveness. When appropriate, overview or introductory chapters are added. Drafts of chapters are peer-reviewed prior to final acceptance or rejection, and manuscripts are prepared in camera-ready format.

As a rule, only original research papers and original review papers are included in the volumes. Verbatim reproductions of previous published papers are not accepted.

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Preface

Nanotechnology has been extolled as one of the key new technologies of the 21st century. Testament to this claim is the integration of nanotechnology into consumer products, which in 2006 encompassed an estimated 212 items. The current inventory (2011) has risen to 1317 products, and by 2020 this number is expected to grow to 3,400 unique products (www.nanotechproject.org). This persistent and not unexpected rate of growth has been met with some trepidation, with concerns centered on unknown ecological and human exposure consequences. Biotechnology, with roots going back several decades, instigated similar concerns when the outcomes and effects of recombinant DNA and genetic engineering were largely unknown. In such cases, the precautionary principle is typically adopted, which states that if an action has a suspected risk of causing harm to the public or the environment, in the absence of a scientific consensus that it is indeed harmful, then the burden of proof that it is *not* harmful (or very unlikely to be harmful) falls on those taking the action. This does not imply that technologies like nano- and biotechnology be banned until they are deemed safe, but rather that an abundance of caution be taken during their implementation and as they mature. This embraces a commitment by the scientific community to acknowledge, understand, identify and model potential risks and hazards. The first chapter in this book, 'Understanding and coping with social risk in emerging technology risk assessment', provides an introduction that scientists and policy makers are often unfamiliar with or, worse yet, choose to ignore. Thompson's discussion of information inequality between parties leading to perceived amplifications of risk is informative reading, and brings home the clear responsibility of scientists and policy makers to effectively and openly communicate with their public constituency. However, this is typically not an easy task considering the extent and complexity of information obtained when dealing with new technologies. Fauss et al. effectively demonstrate the intricacies of information gathering and its structuring into a risk assessment paradigm in their case study of just one nanotechnology input, that of nanosilver. The environmental applications of nanotechnology are discussed by Zhuang and Gentry, hand-in-hand with the associated potential risks that shadow nanomaterials as they intentionally or accidentally enter our soil, aquatic, or atmospheric ecosystems. Zhang and Huang take a more detailed look at nanoparticle properties and more importantly their potential transformations as they interact and react with a suspected major sink for environmental deposition – that of the aquatic ecosystem. The ecotoxicity of fullerene and nanotube materials is discussed by Petersen and Henry, with an emphasis on potential toxic effects in invertebrates, fishes, and plants. Their discussion of experimental artifacts

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contributing to observed toxicities, rather than the nanomaterials themselves, should be duly noted by all researchers participating in the expanding arena of nanoecotoxicology. With much of our ecosystem balance being maintained by its microbial constituency, Ripp delves into the ecotoxicological risks of nanomaterials among these unseen yet crucial members of our environmental community. Finally, Andreescu et al. provide an overview of the experimental methods that make nanomaterial toxicity profiling possible. The information amassed in each of these chapters is extensive but represents only an infancy of our understanding of the totality of nanomaterial risk assessment, and expectedly generates more questions than answers. Nanotechnology, as did biotechnology, will forge ahead prior to knowing all of the answers and outcomes of its potentially transformative advancements. It will be essential that we move forward with an ‘abundance of caution’ and minimal ‘information inequality’.

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Chapter 1

Understanding and Coping with Social Risk in Emerging Technology Risk Assessment

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The experience with global public resistance to so-called GMOs (crops and animals modified using genetic engineering) has become a model for how not to introduce a technology, but one should resist "single bullet" theories of why GMOs have aroused political and social opposition. One contributing cause is that inequalities in access to or ability to utilize information create objectively real vulnerabilities that may be impossible to overcome, at least over a short run. Because biological scientists are relatively rich in information access with respect to genetic technology, they have been slow to acknowledge the validity of risks grounded in information inequality, and have too often engaged in conduct that amplifies these risks. Future programs to pursue genetic technology should be coupled with a serious commitment to better illuminate these vulnerabilities and to develop methods of deliberation, engagement and communication that can mitigate risks arising from information inequality.

Introduction

After almost three decades or research, development and implementation, recombinant DNA modified food crops (colloquially GMOs) have become a paradigm case for the study of public resistance to technology. The consuming public's reaction to these products of biotechnology has been subjected to literally hundreds of different analyses and discussions. Many of these discussions have emphasized risk, and have proposed both measurements of concern about the use

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of this technology, to other agricultural technologies of the 20th century (*1–6*), as well as explanations of why the food consuming public has selectively reacted to this technology (*1*, *7–15*). The sheer number of these competing explanations testifies to the need for a multi-factor approach to understanding the reasons why GMOs became the subject of an international controversy.

The idea of "social risk" that is developed in this paper is intended to offer scientists who work within the conceptual framework of risk assessment a way to integrate findings drawn from the social science literature on GMOs into that framework. Risk assessment can potentially encompass an extremely diverse array of hazards and exposure mechanisms, but as crop biotechnologies began to move through regulatory channels, the legislative mandates for risk assessment within regulatory agencies narrowed the focus of the debate considerably. While this narrowing was, from one perspective, understandable and appropriate, it had the effect of excluding all non-biological hazards from consideration under the rubric of those risk assessments that came to be regarded as definitive both by regulatory agencies and by the scientific community in general. This turn of events became problematic. The problems for GMO acceptance were not caused by the fact that some factors were excluded—any practically useful summary of risks will necessarily be selective. The problems arose because the narrow and exclusionary account developed for biological risk assessment came to be regarded as the definitive account of GMO risks within the regulatory and scientific community. This in turn led to a breakdown in risk management when the scientists most able to speak to reasonable concerns arising outside that community came to regard those concerns as irrational and illegitimate.

The chapter begins by presenting a heuristic for conceptualizing social risks. In succeeding sections, several elements of the heuristic are discussed at some length, in most cases with heavy reliance on previously published analyses. Information inequality is one element of the social risk heuristic that has not been given significant development in the literature on risk amplification or GMOs. As such, this element is singled out for more extensive discussion below. Other elements of the heuristic have been extensively discussed in cited materials and are given relatively little development in this chapter.

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The Social Risk Heuristic

Social risks can be broadly defined as risks, uncertainties, indeterminacies and sources of mistrust that arise in connection with social interactions, and that are subsequently amplified or attenuated by transactions among human beings. In contrast, biological risks associated with hazards such as toxicity or impact on non-target species can often be characterized without reference to social relationships, even when human conduct is a factor in determining exposure or movement of a hazardous substance through the biological environment. Given this definition, much of the literature referenced above dealing with the GMO debate can be interpreted as contributions to our understanding of social risks. Although the term "social risk" is not used widely within this literature, it is a useful construct because it permits one to develop a model of how multiple factors of risk perception,

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social and political processes and structural elements in public policy interact with hazards and exposure mechanisms that are well-characterized by methods drawn from the physical and biological sciences.

The literature from studies of risk perception, risk communication and social amplification of risk (discussed briefly below) has now become so extensive that a simplifying heuristic is a useful way to characterize the relationship between elements of risk that arise in connection with human cognitive abilities and the social situations in which human decision makers find themselves, on the one hand, and bio-physical hazards, on the other. In this paper the term "social risk" is intended to serve this heuristic purpose. The distinction between biophysical risks, on the one hand, and risks that arise in connection with what people believe, know or suspect about the circumstances in which they act has been formulated in numerous ways. The latter were at one time characterized as "perceived risks," but as perceived risks were placed in contrast to "real risks," they came to be thought of as illegitimate, false and therefore dismissable aspects of risk (*16*). A widely read paper by Judith Bradbury described a technical conception of risk utilized in many regulatory risk assessments and contrasted it to risk as a "social construction" (*17*). In my own earlier work, I attempted to characterize a distinction between "probabilist" and "contextualist" concepts of risk (*18*). As Bradbury noted in 1989, risk assessors trained in the bio-physical sciences have often reacted to such proposals for categorizing the sources of risk by presuming that sources in the "other" non-biophyisical category (be it labeled perception, social construction *or* contextual) can and should be ignored. Such sources are viewed as ways of being mistaken about risks. Rather than contest this bias among risk assessors, the social risk heuristic accepts their presumption that biophysical hazards and the conditions of exposure to them constitute the core elements of risk.

Figure 1 illustrates an iterative process that was observed in the GMO controversy, and that has repeated itself with minor modifications in other areas of risk assessment and regulatory decision making. One starts with those factors that might contentiously be labeled "legitimate risks." These include hazards to human or environmental health, and may also include economic hazards (such as loss of income). In any case, these risks are those that have been the traditional focus of risk assessments as they have been performed in public health and environmental science. As is well documented, the measured risks that can be attributed to causes and exposure pathways familiar to physical, biological and economic analysis will be transformed in perception by "heuristics and biases" that are common features of the way that normal human beings make decisions under conditions of uncertainty, incomplete or probabilistic knowledge, (again, these features are given a more detailed treatment below). I have labeled these psychological heuristics as "rational irrationality" because although they may be effective ways to make snap decisions and simplify complex situations, they can lead to errors in specific decision situations. It is rational, in broad sense, to use heuristics that give one "good enough" answers most of the time. However, insisting that these rough-and-ready rules of thumb *define* good decision making would produce inconsistency and incoherence, not to mention poor policy judgments and loss of life (*19*).

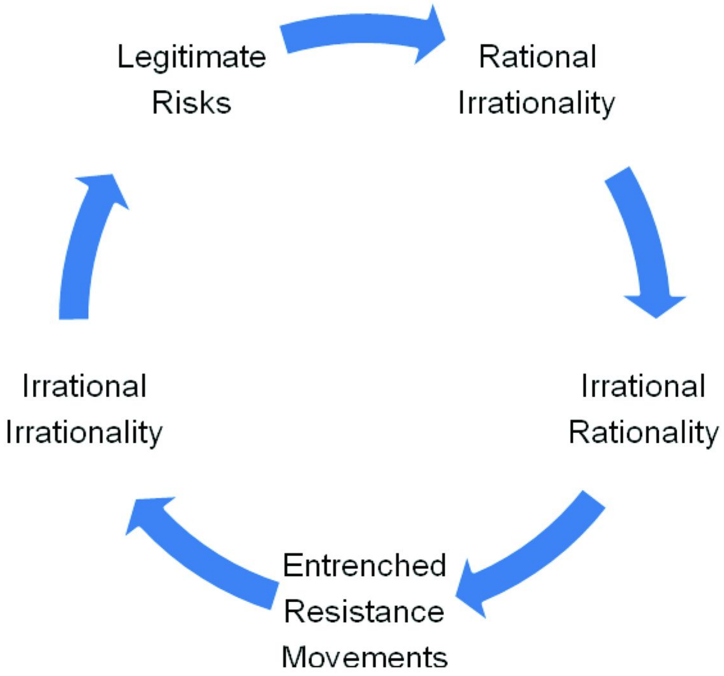

Figure 1. The Cycle of Social Risk.

In addition to these widely studied rational irrationalities it is important to recognize that structural characteristics of information and communication processes can impose risk, even when there may be little evidence for exposure to biophysical hazards that could be derived from classic methods in the natural sciences. This chapter will offer a clarification and examples of such risks below. These might playfully be called sources of "irrational rationality". What might be characterized as imperfections or disfunctionalities in social relationships (in that sense irrationalities) create circumstances in which people quite rationally take themselves to be at risk. Structural inequalities and disfunctionalities, however, have given rise to social movements and to organizations that are dedicated to the eradication or at least the amelioration of these social problems. In the case of GMOs, the existence of these "entrenched resistance movements" created a set of human actors who saw their task as one of opposing both the results or findings of scientific risk assessment, and in some cases the organizations and individuals who developed these findings. As already noted, these entrenched resistance movements have often arisen in response to social circumstances that can quite rationally be characterized as placing certain groups and individuals at risk. It is for this reason that the social risk heuristic, which starts from the assumption that exposure to physical and biological hazards is the source of "legitimate risk" is itself inherently contestable.

There was, however, an additional source of anxiety in the case of the GMO debate. Genes and genetics have become caught up in broad cultural attitudes toward heredity, identity, personal security and the integrity of life. Within this

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kind of cultural environment, eugenic fears that may be quite legitimate in the domain of human biotechnology are generalized and merged into plant and animal biotechnology. They may be expressed in religious language (e.g. playing God) or in the statement that genetic technologies are "unnatural." In the literature on the anti-GMO debates, analysts of the controversy generally take one of two strategies in addressing the role and function of such attitudes toward genetic technologies. Some authors take pains to displace the concerns altogether, showing that they are at best aesthetic preferences that should not be allowed to override agricultural biotechnology's benefits to the environment or to food security (*14*, *20*, *21*). Other authors have re-interpreted these broadly stated anxieties as having their root in one of the domains already discussed. Either they represent reactions that are characteristic of rational irrationalities that have been frequently studied by risk perception scholars (*22*, *23*), or they are inchoate expressions of concern about power inequalities and social structure (*24–26*). Since no one seems to take these concerns at face value, I refer to them as "irrational irrationalities".

However, all of these distortions or (as discussed below) amplifiers for risks can create challenges for communicating what is known about physical and biological hazards. In some cases, these communication problems actually feed back into exposure pathways. Inability to communicate effectively can create unexpected and problematic behavior, in turn creating a situation where ineffective risk communication affects the probability that a hazard will materialize. Thus it is not inappropriate to describe the social risk heuristic as a feedback loop. Given this heuristic, how can risk analysts respond to and accommodate problems that have their origins at nodes of the social risk feedback loop other than those that have been characterized as legitimate risks? Although this chapter cannot provide an exhaustive answer to this question, a few suggestions drawn from the literature of risk studies are discussed below.

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The Potential Scope of Risk Assessment

It is important to recognize that risk assessment is potentially a methodology for anticipating, evaluating and responding a very broad array of possible outcomes or effects that may be associated with the development and dissemination of new technologies. The basic theoretical tools of risk assessment are sometimes traced back to the ancient Greeks, but can be unambiguously recognized in attempts to develop a theoretical analysis of gambling dating back to the 16th century. Quantitative techniques of risk assessment were developed in the insurance industry and were quickly applied to financial investments of many kinds. Risk assessment methodologies were formalized in John von Neumann and Oskar Morgenstern's *Theory of Games and Economic Behavior* (*27*). It was, in fact, comparatively recently that these methods were adapted to the anticipation and management of biological hazards. It is thus somewhat ironic that many specialists in biotechnology risk assessment simply assume that the phrase 'risk assessment' is meaningful within the domain of food safety and environmental impact, but not with respect to social, economic and moral hazards.

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The potential for unwanted social and economic hazards were widely debated when applications of recombinant DNA methods for introducing genetic novelty into agriculturally important plants or animals began to be discussed widely in the 1980s. Cornell economist Robert Kalter published studies predicting the socio-economic impacts of the recombinant animal drug bovine somatotropin in 1985. Kalter's research identified the potential for a structural shift in the United States dairy industry, with farmers milking relatively large herds gaining significant competitive advantages over dairies with small herds (*28*). Although Kalter's findings were later subjected to significant debate, there was little doubt at the time that examining potential changes in the size-distribution of dairy farms was a legitimate application of economic methodologies used to understand the expected costs and benefits of policy change and technological innovation (*29*, *30*). In fact, the study was followed by a number of calls to develop very broad applications of risk assessment to examine impacts on social institutions and power relationships (*31–33*). In such applications, a given social impact, such as a decline the proportion of small to large dairy herds, would be identified as the "hazard," and standard socio-economic methodologies would be utilized to assess the probability that this outcome would materialize. In a policy context, this quantified social risk would simply be weighed in comparison to similarly quantified benefits, such as a reduction in the consumer price of milk. In fact, the Executive Branch of the United States Government conducted a study of social, environmental and food safety risks associated with rBST (*34*). The report noted that U.S. Policy had not, as a matter of risk management strategy, taken such social impacts into account in the past.

The point in the present context is simply that as a tool for predicting and evaluating the potential impacts of new technology, risk assessment is potentially applicable to many different types of outcome. The U.S. Executive Branch's decision on rBST was based on the policy judgment that impacts of this type should not be regulated by the government. It did not imply that social impacts were inappropriate targets for risk assessment. In fact, one can imagine that private firms attempting to determine likely markets and possible political obstacles to the development of products would find it very useful to deploy risk assessment tools to a number of different social risks. However, in contrast to this broad way of understanding risk assessment tools, usage of the phrase risk assessment within the agricultural science community was very quickly constrained to quantification of toxicological and ecological hazards (*35*, *36*). This tendency to ignore non-biological risks led some critics of crop biotechnology to argue that regulatory statutes governing crop biotechnology had created a boundary ensuring that characteristics of fundamental importance to members of the public would be deliberately excluded from consideration (*37*).

The debate, protest and social resistance that attended the introduction of crop biotechnology underlines a class of social hazards that accompany the introduction of new technology. Unlike some of the physical, chemical and biological hazards that have been the focus of risk assessment, many social hazards exist relative to an individual or group's social position. For an investor in new technology, one key social hazard is that protest movements will result in delays or will harm the commercial potential of the technology. For farmers who were users of older crop technology that was displaced by the new technology, social hazards arose in the form of costs that must be born in transitioning to the new technology, but the more significant hazard was bankruptcy as a result of operating amidst larger economic uncertainties created by the transition itself. For those who participate in the protest movement, the social hazard is disenfranchisement, or the usurpation of their right to participate and to have their views reflected in a public policy process. For scientists, the social hazard may be the displacement of their own knowledge and expertise by forms of power or social persuasion that lack backing or standing within the scientific community. In each case, what is a social hazard for one party may be of absolutely no concern for another, and in some cases, one party's social hazard may be another party's social benefit.

The positional relativity of these social hazards suggests that there is no way that a risk assessment could coherently reflect all the potential social hazards that might be associated with the introduction of a new technology. Inclusion of a given social hazard would be highly meaningful to one group, but meaningless or even antagonistic to the interests of another group. Thus although there is nothing in the logical structure of risk assessment that precludes the inclusion of social and economic hazards, there are inherent limitations to using risk assessment as a tool for understanding organizing all the discrete social risks faced by multiple actors in the introduction a new technologies. As long as risk assessment is regarded as a tool for ordinary decision support, this limitation should not be considered to be a defect. A risk assessment will be useful to a decision maker in so far as those hazards of importance to the decision maker have been included; inclusion of irrelevant information will only diminish the usefulness of the analysis. However, the function and purpose of risk assessment has expanded considerably beyond that of decision support over the past forty years. As early as the publication of the Nuclear Safety Report in 1975, risk assessments have been represented as documents that report risks of relevance to the general public objectively and impartially (*38*). But as John Dewey argued in 1927, there is no *general* public, there are only multiple publics, each with different interests and needs (*39*). There is no way to reconcile positional social hazards in a document represented as an objective and impartial report on risks of relevance to the public at large.

This suggests that simply expanding risk assessments so that they include analysis of outcomes that would be regarded as adverse by some segment of the public, but not other segments, is not a promising approach to addressing social risk. Some observers of the GMO debate have recommended precisely this approach (*40–42*). Offering an extended rebuttal of this proposal would take the present enquiry far off track, so the above remarks on multiple publics and the positional relativity of social risk must suffice. The heuristic of social risk sketched in the opening section of the chapter provides an alternative that allows regulators and the scientific community to acknowledge that social hazards do indeed contribute to the risks that are borne by people occupying a given social position. This seemingly simple acknowledgement might well have changed the tenor of the debate over GMOs. By implying—and in some cases explicitly arguing (*43*)—that social hazards have no standing in rational assessments of risk from emerging technologies, the voices heard from the Science and regulatory

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community created a situation in which *all* risks, not just social risks, were subject to a process of amplification.

The Amplification of Risk Paradigm

The social risk heurist sketched above draws implicitly on the "social amplification of risk" paradigm, which arose initially among social scientists researching the perception of risk. In 1988, a distinguished group of risk scholars presented a framework for understanding how physical, chemical and biological analyses of risk conducted under the auspices of risk assessment interact with various psychological and social factors to influence the perception of risk, and subsequent events associated with political and behavioral responses to risk. Succinctly, it suggested that people are prone to regard comparable quantitative estimates of hazard and exposure as more or less risky depending on a host of socio-political and psychological factors. The amplification of risk framework was intended to organize and help communicate a large and disparate body of social science research that had been accumulating for some time, including studies on the psychology of quantitative decision making, rhetoric and persuasion, and the sociology of trust (*44*).

The social amplification paradigm has had a prominent place among proposed explanations for the public's reaction to crop biotechnology and GMOs. Authors have attributed the public's reaction to factors such as "outrage" (*45–47*) and to the role of the mass media in leading people to view the risks of GMOs as significantly greater or more threatening than the commonly-held view among scientists would suggest (*48–50*). In reviewing food safety concerns from biotechnology, Yeung and Morris note a number of factors often associated with the risk amplification framework: risks that are involuntary are amplified, while those that are voluntary are attenuated; risks to which many are exposed are amplified, while risks borne by few are attenuated; and risks with delayed hazards are amplified, while risks with whose outcome is known quickly are attenuated. They note that each of these characteristics applies to foods from crop biotechnology and conclude that risk amplification explains why the public's reaction to biotechnology is inconsistent with a risk assessment based on chemical and microbiological food safety hazards (*51*).

However, the underlying epistemic and ontological questions raised by the amplification of risk paradigm have not been the focus of a great deal of attention in risk studies. Some socio-psychological factors associated with the amplification of risk have almost universally been understood to involve errors in judgment, or more pointedly *mis*perceptions of risk. Factors associated with the cognitive processes of anchoring and framing would be prime examples of amplifiers that are thought to have no legitimate role in altering the circumstances that characterize risk in a given situation. Anchoring is a well documented psychological tendency toward bias in judgment (especially quantitative judgment) due to the way that information is recalled and utilized in the decision making. Particularly memorable bits of information are more likely to sway judgment than less memorable (but potentially more relevant) information.

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In fact, information presented early in sequence has been shown to influence subjects' response to information presented later in the sequence, even when subjects understand that there is no logical relationship that would warrant such an inference (*52*). Framing is a similar phenomenon, where subjects offer differ solutions to quantitatively identical problems depending on qualitative descriptors (such as whether a choice is presented in positive or negative terms) (*53*).

In other cases it is far less clear that the social factors leading to an amplification of risk should be regarded as leading people to make erroneous or faulty judgments. Circumstances in which risk is amplified because of a subjects' distrust of other actors provide a particularly important class of examples. An actor's competence or willingness to take appropriate precautionary actions is of obvious and undeniable relevance to the likelihood that hazardous outcomes will, in fact, materialize under a given set of conditions. Estimating such probabilities quite properly takes account of such factors when they are introduced into standard risk assessment. Social factors that influence trust in an actor's competence or intentions are often simply situation-specific features that bear on this estimate in ways that a more generic risk calculation is unable to accomplish. An example can illustrate this general point.

In a given traffic situation, a driver may be presented with a number of different types of evidence that will shape their confidence in the competence or attitudes of other drivers, including behavior such as aggression, talking on a cell phone, general inattention or in some cases, simply driving a certain kind of car. Distrust in other drivers exhibiting these traits is both appropriate and a perfectly rational amplifier of the standard risk calculations that are made to estimate the probability of a motor vehicle accident. It would be irrational *not* to see such situations as "more risky" than the typical case. This is nothing more than an informal self-assessment of risk performed by the driver, and it is in total accord with standard procedures of risk assessment. Yet these self-assessments have absolutely no bearing on national statistics or estimates of motor vehicle accident risk. Risk calculations have been made for various automobile transportation activities for several decades. It is not as if the official motor vehicle accident risk estimates need to be recalculated every time someone sees a fellow driver that they feel they cannot trust!

What this example illustrates is that some phenomena included in the umbrella of risk amplification (or attenuation) are, within a sufficiently well-defined problem setting, totally appropriate indicators for exposure to hazards. This has led some contributors to the risk amplification literature to argue against the tendency to characterize risk amplification as contributing to an erroneous or misleading perception of actual risk (*54*, *55*). Writing specifically on GMOs, Gaskel and coauthors argue that the amplification factors that have influenced public reaction are in fact all quite rational, relating specifically to lack of trust in key actors such as regulators and the food industry (*56*). In summation, while attention to social risks and risk amplification provides a useful starting framework for analyzing social risk within the context of risk assessment, it is critical to address the fundamental question of whether amplifiers are actually amplifying risk, or whether they are contributing to a misperception of risk. One

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advantage of the social risk heuristic presented in this chapter is that it provides a way to acknowledge and address this problem.

The example also reflects the possibility for structural social relationships that quite rationally contribute to the riskiness of a given situation. In this, it exemplifies a set of characteristics that have not been widely incorporated into the social amplification of risk paradigm. However, other and older studies from institutional economics provide a clear theoretical basis for showing how at least some structural features of social relationships can contribute to risk.

Information Inequality as a Source and Amplifier of Risk

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In one of the seminal papers in institutional and behavior economics from the 1970s, George Akerlof introduced the concept of quality uncertainty as a source of market failure. Akerlof discussed how the price of used cars reflected buyers' concern about the risk of purchasing a "lemon", the then common colloquial term for a car plagued by atypical quality defects leading to higher than average costs for maintenance and repairs. Lemons represent a classic case of high-consequence/low probability risk, an outcome on the tail of the risk distribution that skews the potential risk taker's evaluation of the potential hazards associated with a given activity. In the case of used cars, there was a widespread perception that frequency of repair was not evenly distributed across the population of all cars, and that certain cars—lemons—were, for unexplained reasons, far more likely to need seemingly unrelated expensive repairs. Quite naturally, owners of these high maintenance vehicles were more likely to make them available on the used car market. The existence of these cars in used car markets created a high degree of uncertainty among buyers about the quality of any used car that they were considering for purchase, leading to a tendency for the price of all used cars to be lower than economic theory would otherwise predict (*57*).

Akerlof's choice of the term 'quality uncertainty' to account for this economic phenomenon was, in certain respects, infelicitous. The term implies that consumer's uncertainty about the quality of the car they were about to purchase was the source of their risk averse economic behavior. But purchaser's of new cars also face the chance of acquiring a lemon. In a strict sense, quality uncertainty is the same. However, there is presumably no objective basis for distinguishing between a lemon and a car more in line with average quality expectations on the new car market. New car dealers had no prior experience with repair and maintenance of their vehicles. Thus the reason Akerlof chose to analyze used cars is that sellers possess information about the vehicles that buyers lack. The used car market violates one of the assumptions taken to be characteristic of efficient markets when Akerlof wrote: all parties to an exchange possess full information. It thus appeared to be an interesting and challenging case for economic analysis. In part, the reputation of Akerlof's work among economists rests upon his ability to provide a compelling economic analysis of a market that manifestly violated one of the standard constraints of economic theory.

For present day scholars of risk, the fact that parties to the used car transaction possess different sets of information is far more significant than quality uncertainty. It is the inequality of information between buyer and seller that makes the purchase of any used item, especially one involving the potential for hazards not immediately apparent on simple inspection, inherently risky. Indeed, the significance of Akerlof's paper among economists is widely seen in terms of his willingness to relax the assumption of equal information that had been a fundamental tenet of neoclassical welfare economics (*58*). Information inequality shows how structural aspects of the way that people are situated with respect to one another can create circumstances of risk that it would be irrational to deny or fail to acknowledge. Although Akerlof's paper is rarely cited by scholars working in the social amplification paradigm, it is clear that the existence of inequality in access to information between parties would be seen as a positional amplifier of risk. That is, legitimate risks would quite rationally be seen as amplified by those who are (or rationally take themselves to be) in the position of lacking information that other parties possess.

What is more, information inequality provides a clear example of a structural feature that would amplify risks in the manner suggested by the "irrational rationality" category in the social risk heuristic discussed above. Information inequalities represent social irrationalities, that is, disfunctionalities that *create* circumstances of vulnerability and risk even where they might otherwise not exist. Akerlof's paper presumes that markets would function to distribute the inherent or "legitimate" risks in purchasing a used car. Hazards of breakdown and failure could, will have distributions that reflect the total population of used cars. Were those used cars that are for sale a fair sample of that total population, the price of a used car would reflect what buyers are willing to bid given that risk. However, because sellers know something that buyers do not, the sample of cars for sale is not representative. In fact, in the years since Akerlof's article was published the structure of the market for used cars has evolved dramatically, with services providing mechanics' inspections, repair records based on VIN numbers and statistics compiled by organizations such as Consumers Union. All of these structural responses to information inequality have made purchasing a used car considerably less risky than it was in 1970. The structural environment for used car purchases has, in that sense become rationalized over time in response to the irrational rationality created by information inequality.

Although few analysts appeal to information inequality in explaining public reactions to GMOs, many of those who appeal to the role of trust in the debate are in fact noticing that companies and to some extent all scientists possess information that the public, especially vulnerable parties, lacks. Studies of consumer perceptions often noted concerns about which information to trust (*59*)(*60*). Aerni and Bernauer, for example, studied farmer and consumer reactions in the Phillipines. While these stakeholders were not initially predisposed to resist agricultural biotechnology, competing narratives on biotechnology created a situation in which they felt that they were not in a position to judge which risks are legitimate and which are not. In this situation, their lack of information that they could deem reliable created a vulnerability that was articulated as lack of trust in those purveying the technology (*61*). Thus information inequality does

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provide a conceptual framework for understanding risk perceptions associated with GMOs.

Conclusion: Information Equality Social Risk and the Social Position of Scientists

This chapter has presented a heuristic device for conceptualizing the relationship between social risks and the physical or biological risks that are the more typical focus of scientific risk assessment for emerging technologies in food and agriculture. Biotechnology and nanotechnology are two such technologies. While classical health and environmental hazards determine a category of “legitimate risks”, the degree to which people see technologies as “risky” will also be affected by amplifiers of various kinds. Among these social amplifiers, those that reference the way that people take themselves to be at risk as a result of their structural vulnerability to exploitation, harm or deceit on the part others are particularly insidious. Their pernicious character is in part due to the existence of entrenched resistance movements. Organizations dedicated to the elimination of social inequalities and injustices quite naturally and quite properly view technical innovations that exacerbate or perpetuate such vulnerabilities with a jaundiced eye. The existence of these movements ensures that human actors will respond to innovator’s attempts to promote their technologies with actions that are intended to have just the opposite result (*62*).

However, such responses would not be characterized as insidious or pernicious were it not for the fact that the vulnerabilities themselves represent “social irrationalities”: dysfunctional social institutions that create or amplify risks to no one’s benefit. Yet it is important to underline the fact that when such social irrationalities exist, vulnerable parties (and their agents) are *entirely* rational in perceiving themselves to be at risk and responding accordingly. This chapter has shown how the social amplification of risk paradigm and literature from institutional economics on information inequality can be used to provide a theoretically rich picture of the social risk heuristic. When nodes of the social risk heuristic characterized by structural or information inequalities act in concert with more widely studied elements of risk perception and with cultural attitudes toward genes and genetic technologies, the potential for miscommunication and misunderstanding of risk assessment results is significant.

The chapter has not explored ways to modify or reform formal or regulatory risk assessment methodologies to more adequately cope with the challenges of social risk in any detail. In that sense, expectations created by the title of the chapter may have been disappointed. Yet surely the first stage in coping with these problems is simply problem formulation. What is more, the record of institutional response to the problems identified by Akerlof in 1970 suggests a line of research that would, in fact, develop coping mechanisms. This research would examine how the settings in which people access information about risks from emerging technology might be made more equitable, and how positional risks might be addressed by changes in policy and practice. In the interim, the least that scientists and engineers can do is resist the temptation to disparage responses to information

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or power inequalities that are both rational and appropriate simply because they do not cite or reference hazards that have their roots in physical and biological systems. That is, in an important sense, the first step toward coping with social risk.

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Chapter 2

Case Study of an Emergent Nanotechnology: Identifying Environmental Risks from Silver Nanotechnology through an Expert Elicitation Methodology

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Environmental risks posed by silver nanotechnology products are identified through an expert elicitation process to judge the relative likelihood of occurrence of pre-identified exposure scenarios and severity of particular hazard factors. The intersection yielded a ranking of the environmental risks for various product types. Colloidal nanosilver products were found to pose substantial risks within aquatic environments. Bio-accumulation of nano-silver was judged as a significant factor causing chronic exposure. The risks are delineated within the product lifecycle using product maps. In this manner, risks can be identified prospectively to enable adaptive management of scientific innovation and regulation.

Rationale

The current literature on toxic effects of nanomaterials involves a small number of studies that do not yet yield conclusive results; and it may take a decade or so to develop the rigorous scientific data and understanding needed by regulators, who rely on quantitative risk assessment. At the earliest stages of technological development, risks and benefits are not known or quantifiable, and the law of unexpected consequences rules (*1*). An alternative more favored in Europe than the US is the precautionary principle. Whereas risk methodology requires that harm be proved, the precautionary principle requires that safety be

In Biotechnology and Nanotechnology Risk Assessment: Minding and Managing the Potential Threats around Us; Ripp, S., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2011.

proved; which is impossible for emerging technologies. A premature moratorium on new technologies not only blocks potential risks, it also blocks potential benefits. Silver nanotechnology (nano-silver) has emerged as the most prevalent use of nanotechnology within consumer products today, according to the Project on Emerging Nanotechnologies Consumer Product Inventory (*2*). It is used as an antimicrobial in products ranging from cleaning sprays and no-odor clothing to filters for drinking water. While silver is an element that is familiar to the public and has been regulated for over a century, its usage in the nanoscale form presents an especially interesting case study (*3–5*). While the disinfection mechanism of silver has been hypothesized to arise due to the release of Ag^+ species which can interact with thiol groups of vital enzymes to affect cell metabolism, the ability of DNA to replicate, and disrupt bacterial cell membranes, an important additional contribution at the nanoscale arises from enhanced catalytic action at nano-Ag surfaces which results in the release of reactive oxygen species (ROS) (*6*, *7*). Its application within products as nano-silver is motivated by the ability to modulate, sustain and target the disinfection action at a molecular scale, as well as integrate disinfection with other functions, such as sensing and controlled release. However, potential problems include the attrition and run-off of free silver nanoparticles (Ag-nps), their action on beneficial micro-organisms that perform key ecological functions, and effects arising from their broader bioavailability such as the rise in silver resistance amongst microbial species (*8*, *9*). These environmental implications are highly dependent on the size, shape, and functionalization of nano-silver as well as the environmental conditions of the water samples they are in contact with (*10*, *11*). It is hence of great interest to identify the substantive risks and relate them to the nano-silver products currently in the market. In this paper, we aim to conduct this through an expert elicitation methodology to explore the intersection of pre-identified exposure scenarios with inherent nanoscale material properties that enhance the risk potential, henceforth called *exposure factors* and *hazard factors*. In this manner, the expert elicitation framework can be applied to judge the relative likelihood of occurrence and severity of particular environmental risks. The methodology also allowed for a mapping of the risk "hotspots", identification of the knowledge and regulatory gaps, identification of impacts from the risks, and could eventually aid in the formulation of dose metrics that need to be monitored to mitigate the risks. This can provide the basis for proactive risk-based EHS regulation involving a form of adaptive management, in which particular risks can be identified and EHS procedures can be adjusted as methods and technological frontiers advance.

Challenges in Prospective Risk Identification

The maximum degrees of freedom for managing an emerging technology occur at the design and development phases. Earth Systems Engineering Management (ESEM), a set of principles that grew out of industrial ecology, forms the basis of our work (*12*, *13*). At its core is reversibility; where new technological systems are designed so that they can be modified or even shut down if unanticipated negative impacts emerge. Risk assessment, in contrast, works best after a technology has been in use for a period that allows for quantification of risks. At this later stage of development, the industry may be locked into systems that are difficult to reverse. For example, products using silver nanotechnology are entering the marketplace with no systematic data on their effects and a regulatory system poorly adapted to manage them. This means that the product developers themselves could be blind-sided by an incident that raises public concerns, whether these concerns are legitimate or not. Identification of risks from nanotechnology is already a topic of much interest (*14*, *15*). Hence, risk identification is essential to, and the first major step in, traditional risk assessment. This leads to risk filtering, ranking, and management (*16*). Early identification of potential risks can also assist in prioritizing EHS research when budgets are constrained for such research. In this manner, potential risks can be given more attention relative to an exhaustive but undifferentiated list. Finally, adaptive management using ESEM requires the capability to identify risks early and adapt the EHS policy to accommodate the needed changes.

A primary challenge for prospective risk identification is the scarcity of information on nanomaterials and nanotechnologies, including information on how the size, shape, chemical composition and catalytic properties of nanoparticles interact with human and environmental systems. This is accentuated by the lack of standardized indices to judge toxicity, knowledge of dose metrics to mitigate exposure, and the absence of a systematic nomenclature for nanomaterial components. A secondary issue arises from enhanced exposure, since nanoparticles can be easily transported through the ecosystem and there are as yet no means to monitor them continuously, in real-time. Finally, there are system-level human health and environmental risks, since nanoscale modifications of inherent material properties can lead to non-localized system-level impacts.

Regulatory Perspective on Silver and Nanosilver

To understand where silver nanotechnology fits in the regulatory structure today, it is important to understand how silver has been regulated in the past and what mechanisms the government has to control silver nanotechnology in consumer and commercial products. The Environmental Protection Agency (EPA) regulates silver as a chemical under the Toxic Substances Control Act (TSCA) and as a pesticide under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (*17*). Under TSCA which regulates new or already existing chemicals, silver is listed as a registered "chemical substance," CAS# 7440-22-4. Table 1 shows the U.S. regulatory exposure limits. Currently, the EPA has permitted limited manufacture of new chemical nanoscale materials through the use of administrative order or Significant New Use Rules (SNUR). For silver to fall under this regulatory net, the use of the nanosilver in a particular product would have to be considered to be significantly new use. However, new rules that EPA proposed to apply by the end of 2010 included:

1) Formalizing a SNUR under section 5(a)(2) of TSCA that would require people who intend to manufacture, import, or process certain nanoscale materials for a significant new use activity to submit a Significant New Use Notice (SNUN) at least 90 days before commencing that activity;

2) A rule that would require the submission of additional information of nanoscale materials already in commerce under TSCA section 8(a);
3) A rule that would allow the EPA to require additional testing of nanoscale materials of interest already in commerce under TSCA section 4 (*18*).

Table 1.

OSHA & NIOSH Exposure Limits for Silver Metal and Soluble Compounds[1]		
OSHA Permissible Exposure Limit	TWA 0.01 mg/m^3	
NIOSH Recommended Exposure Limit	TWA 0.01 mg/m^3	
TWA is the total weight average over an 8 hr period. The United States Department of Labor Occupational Safety and Health Administration (OSHA) and the National Institute for Occupational Safety and Health (NIOSH) regulate silver in regards to human exposure in the workplace. This includes forms of exposure such as dermal absorption, ingestion, inhalation and chronic effects. OSHA has a Permissible Exposure Limit (PEL) of 0.01 mg/m^3 TWA (8hr total weight average) for silver metal and soluble compounds for general industry.[2] NIOSH has the same limit of 0.01 mg/m^3 TWA for their Recommended Exposure Limit (REL) for silver metal dust and soluble compounds. NIOSH also lists the Immediate Dangerous to Life or Health Concentrations (IDLH) level as 10 mg/m^3.[3]		
EPA Exposure Limits on Silver		
National Secondary Drinking Water Regulations[4]	0.1 mg/L	
Oral Reference Dose (RfD)[5]	0.005 mg/kg/day	
Current National Recommended Water Quality Criteria, Priority Pollutants Criteria Maximum Concentration[6]	Freshwater	3.2 µg/L
	Saltwater	1.9 µg/L
Effluent Limitations Guidelines for the Centralized Waste Treatment Point Source Category[7]	Daily maximum	0.12 mg/L
	Monthly maximum average	0.0351 mg/L

Continued on next page.

Table 1. (Continued).

The EPA also regulates the amount of silver. For drinking water the EPA has silver listed under secondary standards, which are non-enforceable guidelines that regulate contaminants that can cause cosmetic effects. The Integrated Risk Information System (IRIS) provides health assessment information on a chemical substance such as an oral reference does, once a comprehensive review of chronic toxicology data has been completed by U.S. EPA health scientists. The Clean Water Act (CWA) regulates the discharge of materials to the sewer through the EPA's Effluent Guidelines. And it is also regulated under the hazardous waste program in conjunction with the Resource Conservation and Recovery Act (RCRA).[8]
The National Recommended Quality Criteria lists silver as a "Priority Pollutant" the above limits are determined for the "Criteria Maximum Concentration" (CMC) which is an estimate of the amount of silver in the surface water to which an aquatic community can be exposed briefly without resulting in an unacceptable effect. The EPA also lists "Criterion Continuous Concentration" (CCC) for various pollutants, which is the highest concentration of a material that an aquatic community can be exposed indefinitely without resulting in an unacceptable effect. They do not provide a CCC concentration for silver.[9]
The EPA provides guidelines for effluent levels of silver for centralized waste treatment centers. These regulations would be applied to sewage treatment plants that would treat wastewater. The daily and monthly maximums are listed under what is known as Best Practicable Technology (BPT) limitations or the best available technology for pollution control at a reasonable cost for implementation and operation under normal conditions.

[1] National Institute for Occupational Safety and Health. (2005, September). [2] U.S. Department of Labor Occupational Safety & Health Administration. (2006, August 5). *Silver, Metal & Soluble Compounds (as Ag)*. Retrieve June 23, 2008, from Chemical Sampling Information Online, from http://www.osha.gov/dts/chemicalsampling/data/CH_267300.html [3] National Institute for Occupational Safety and Health. (2005, September). *Silver (metal dust and soluble compounds, as Ag)*. Retrieved June 23, 2008, from NIOSH Pocket Guide to Chemical Hazards Online, from http://www.cdc.gov/niosh/npg/npgd0557.html [4] U.S. Environmental Protection Agency. (n.d.). *Drinking Water Contaminants*. Retrieved June 23, 2008, from http://www.epa.gov/safewater/contaminents/index.html [5] U.S. Environmental Protection Agency. (1996). Silver (CASRN 7440-22-4). Integrated Risk Information System. Retrieved April 5, 2010, from http://www.epa.gov/IRIS/subst/0099.htm [6] U.S. Environmental Protection Agency. (n.d.). *Current National Recommended Water Quality Criteria.* Retrieved June 23, 2008, from http://www.epa.gov/waterscience/criteria/wqctable/#cmc [7] U.S. Environmental Protection Agency (2000, December 22). Effluent Limitations Guidelines, Pretreatment Standards, and New Source Performance Standards for the Centralized Waste Treatment Point Source Category. (Volume 65, No. 247, DOCID:fr22de00-25). Retrieved June 27, 2008, from Federal Register Online via GPO Access: http://www.gpoaccess.gov/index.html [8] U.S. Environmental Protection Agency. (1986, June 26). *Silver in Wastes and in Sewer Discharges from the Photo-finishing Industry* (RCRA Online No. 12674). Retrieved June 23, 2008, from RCRA Online Access: http://www.epa.gov/rcraonline/ [9] U.S. Environmental Protection Agency. (n.d.). *Current National Recommended Water Quality Criteria.*

As of mid-2011 EPA has only made progress on the first of these new rules. In June 8, 2010 a direct final rule was declared on the Significant New Use Rule on certain cemical substances (75 Fed. Reg. 35977) (*19*). The EPA issued the first nanotechnology SNUR to multi-walled and single walled carbon nanotubes in October 18, 2010 (*20*). They have yet to add a rule for silver nanoparticles. Through FIFRA, the EPA regulates the sale, distribution and use of "pesticides." However, regulation of a product is largely determined by the claims made for the product when it is sold or distributed. Products claiming to kill pests or prevent the growth of pests and products claiming to protect inanimate objects from the harmful effects of pests are considered to be pesticides (*21*). The use of silver in most consumer products falls under the FIFRA pesticide classification of "antimicrobial pesticide." The data requirements for registration of a pesticide largely depend on the intended use of the product. These studies can include product data requirements for product chemistry, toxicology, ecological toxicity, human exposure, environmental fate and residue chemistry (*22*). Additionally, silver can come under the purview of the Federal Food, Drug and Cosmetic Act (FFDCA) sec. 408 which states that any pesticide chemical residue in or on a food (including animal feed) shall be deemed unsafe unless the residue is within a tolerance limit or it is exempt from the requirements of that tolerance (*23*).

In summary, FIFRA leaves the EPA with significant regulatory power with regards to regulating silver nanotechnology as a pesticide. Furthermore, the new proposed rules to TSCA give the EPA the ability to make new demands on nanotechnologies already in commerce. The pertinent question then becomes: is silver nanotechnology a significant new use of silver and does it pose a new unique risk as a nanomaterial based pesticide?

Industry Perspective on Nanosilver Regulation

Representatives from industry in the Silver Nanotechnology Working Group (SSWG) suggest that from a historical perspective the use of nanosilver is not new and does not pose a new unique risk. A cogent argument can be made to support their claims. The first silver colloids were registered in 1954 with the EPA as algaecides and silver-impregnated carbon filters were widely used in the 1960's to protect municipal water supplies. In this regard the use of silver in the treatment of water is not uncommon and these types of products have not caused significant problems. It is estimated that 82% (75 of 92) of silver pesticide products registered with the EPA already contain nanoscale particles or ionic silver (*24*). These results suggest that the use of nanosilver as an antimicrobial is not a significant new use of silver.

SSWG's second claim is that nanosilver, when used as a pesticide does not pose new risks. This is based on the assumption that the predominant antimicrobial mechanism of the silver nanoparticle is the release of the silver ion and that the toxic effects of nanosilver are proportional to the rate of release of free silver ions. Wijnhoven identified this as the 0-hypothesis (*25*). If true, then risks of silver nanotechnology can be equated to that of the risks of the silver ions it releases. The toxicity of the silver ion is well defined due, in large part, to work done to determine the environmental effects of silver ion contamination resulting from silver mining operations and photography processing plants (*26*)(*27*). Limited work has been done to look at the release rates of silver ions from nanoparticles. Some prior work suggests the importance of Ag ion release in 20nm polymer coated spherical nanoparticles and citrate capped silver nanoparticles in aqueous environments, thereby attributing primary disinfection action to silver ion release rates (*28*, *29*).

It is in the best interest of applicants trying to register products under FIFRA to follow the so-called 0-hypothesis. If toxicity of nanosilver is in fact proportional to silver ion release only, then this would remove many of the substantial data requirements that the EPA places on manufacturers, importers and distributors of silver nanotechnology antimicrobial products. However, there are a handful of other groups that claim nanosilver toxicity is due not only to ion release but also to the generation of reactive oxygen species (ROS) or free radicals and the direct interactions of the silver nanoparticle with bacterial structures (such as the membrane and DNA). Kim et al. (*30*), Morones et al. (*31*), and Navarro et al. (*32*) in exploring the antimicrobial effects of silver nanoparticles concluded that disinfection rate could not be explained by silver ion release alone. While Liu's group thought the silver ions were the primary disinfection mechanism they also detected the presence of hydrogen peroxide, a ROS and attributed it to an uncharacterized oxidation mechanism. These results suggest that the 0-hypothesis could be incorrect and that more research into the disinfection mechanism of nanosilver is needed.

The Need for Regulatory Guidelines

This leaves the EPA in a state of uncertainty on how to appropriately regulate silver nanotechnology. In 2007 over 240 silver nanotechnology products were being sold to both consumer and commercial markets (*33*). Guidelines are needed to help applicants for product registration, so that they can understand what data requirements are necessary and to ensure that nanosilver enhanced products are used in a way that does not pose environmental or human health issues. To determine what are the important factors in nanosilver regulation the EPA has reached out to various experts and organizations for help. To date, the EPA in collaboration with various groups (nationally and internationally) develops guidelines for both nanosilver and nanomaterials. The Organization for Economic Co-operation and Development (OECD) has established an international Working Party on Manufactured Nanomaterials (WPMN) to help understand the risks of nanomaterials. Silver has been identified as one of the manufactured nanomaterials for environmental health and safety testing. NIST and the Army Corps of Engineers held a workshop on nanosilver in April 2009 in Mississippi with the goal to establish criteria for the selection of materials for the OECD nano-silver testing program. Within the EPA itself, the FIFRA Scientific Advisory Panel met in November 3-6, 2009 in Arlington, VA to discuss issues associated with nanosilver and other nanometal pesticides (*18*, *21*, *22*).

As of November 2009 the EPA's view of nanosilver is that "the current state of science does not contain sufficient information to determine definitively whether (and, if so, to what extent) various forms of nanosilver particles may cause toxic effects beyond those attributed to the release of silver ions" (*22*).

Risk Identification Using an Expert Elicitation Methodology

A primary challenge for regulators and decision makers is to enable mechanisms that can broadly identify risks within emergent technologies and implement policies to mitigate damages and provide clear guidelines to businesses. These new technologies present many economic opportunities for businesses. In the case of nanosilver hundreds of products are currently on the market. Nanosilver is relatively easy to fabricate and can be incorporated into plastics, textiles, coatings, liquids and powders. However, businesses can be wary to implement these new technologies if there are no clear regulatory guidelines. Ultimately they do not want to produce goods that will be recalled, fined or increase costs due to excessive safety testing. For example, IOGEAR, an electronics company, was fined $208,000 for making antimicrobial claims on silver and titanium dioxide coated "Germ Free" keyboard and mice peripherals (*34*). To help minimize harmful environmental contamination and human health risks it is in the best interest of regulators, business and the public for the implementation of anticipatory governance, where risks can be identified early on, and decision makers can direct funds to the areas of most immediate concern. This coupled with adaptive management in which an iterative process will allow new information to be integrated into policies and funding decisions would create a flexible regulatory model, able to adapt more readily to emergent technologies. Hence, in this section we review some highlights of results on risk identification using an expert elicitation methodology.

Methodology

Since Ag nanoparticle dose metrics and data about particular risks are scarce for the application of traditional risk assessment methods; our framework builds on expert opinion and existing research and data, as depicted in Figure 2, based on an earlier method used to compare various nanotechnology products (*35*). In this study, information was initially gathered through the creation of a Silver Nanotechnology Commercial Inventory and a literature review. Data collected from these studies were used in an expert elicitation as seen in Figure 1. An interactional expert interviewed a panel of 10 experts with a variety of experience and academic backgrounds, as summarized in Table 2. In separate private interviews conducted by the interactional expert, the current product information derived from the creation of a Silver Nanotechnology Commercial Inventory (SNCI) was provided to each expert. Each expert was engaged in a discussion to help identify and rank the relevant exposure scenarios and inherent material characteristics (physico-chemical properties, reactivity and toxicity), that were classified as hazard and exposure factors. The interview transcripts were analyzed by the interactional expert, as well as by another research group member to independently rank the exposure scenarios and risk triggers. The exposure component of the risk was estimated from the exposure scenarios, and the hazard component of the risk was estimated from the hazard-based risk factors. Exposure was plotted against hazard to eventually get information on risk hierarchy for the different silver nanotechnology applications. We focused chiefly on identifying, distinguishing, and classifying the risks from coatings, colloids and powder product applications. Information was also collected on knowledge gaps to help focus pertinent research towards key issues for evaluating the technology and understanding regulatory issues, and thereby provide a context to some of the challenges that face nanotechnology. The methodology is detailed in prior work Fauss et al. (*36*), and we focus herein on the results and their implications.

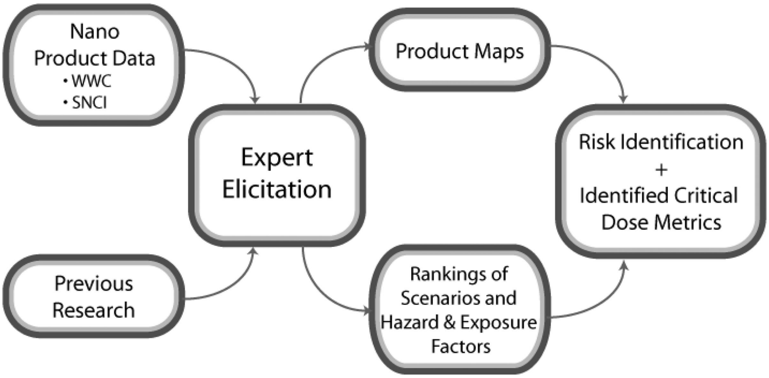
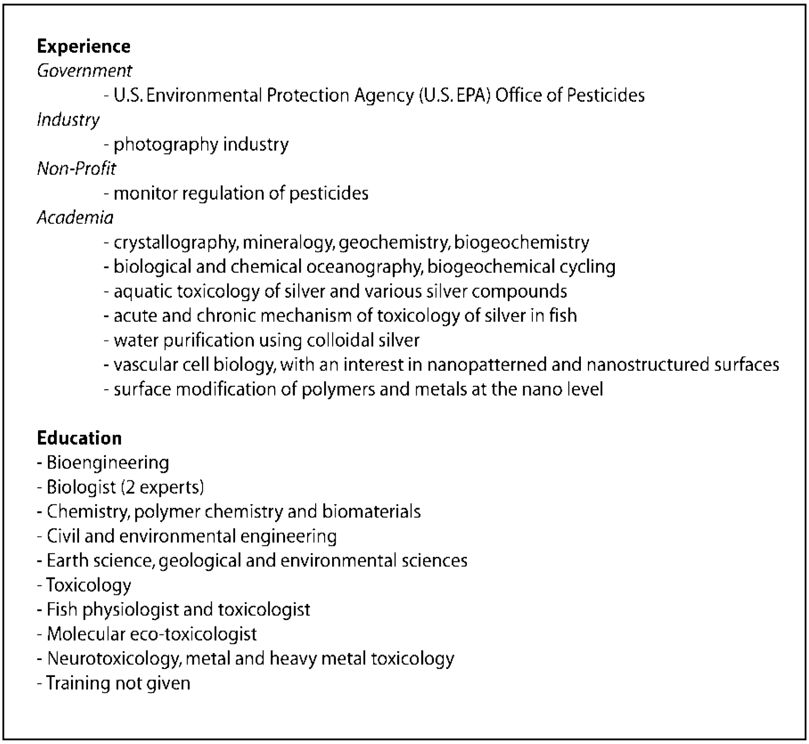


Figure 1. Expert Elicitation Methodology. Above is the overview of the expert elicitation method that leads to risk identification of emergent technologies. Information from the Silver Nanotechnology Commercial Inventory together with data from a literature review were included in the expert elicitation of the 10 experts. From these interviews data was mined from the transcribed discussions in the form of product maps, rankings of exposure scenarios, hazard factors and exposure factors. Together these help to identify and rank the risks and benefits of the emergent technology along with identifying those factors that are critical in determining and measuring that risk.

Results

Risk is composed of two components: exposure and hazard. It is important to consider both these factors when evaluating the risk of silver nanotechnology. During the expert elicitation process the experts helped to identify exposure and hazard risk factors. Exposure risk factors (Table 3) are those factors that are critical in characterizing the exposure of nanosilver to humans, micro-organisms or the broader environment. Hazard risk factors are those that contribute to the potential hazard of nanosilver. These can be divided into three categories that contribute to a hazard assessment: material properties (Table 4), material reactivity (Table 5), and material toxicity (Table 6). Experts also identified exposure scenarios (Table 7) that should be considered in the evaluation of silver nanotechnology. At the time of this expert elicitation in 2007-2008, there was only minimal work published on exposure factors (material properties that enhance nanosilver exposure), hence, experts would not commit to ranking the list. The study in this portion of the research was therefore focused on understanding the specific concerns for each of these factors and the associated knowledge gaps. On the other hand, based on a greater availability of data, experts did rank the exposure scenarios and hazard related factors, hence, this was used to plot the net risk posed by each nanosilver product type (coatings, colloids, and powders).

Table 2. Experience of Experts Involved in the Elicitation Process



Experience

Government

- U.S. Environmental Protection Agency (U.S. EPA) Office of Pesticides

Industry

- photography industry

Non-Profit

- monitor regulation of pesticides

Academia

- crystallography, mineralogy, geochemistry, biogeochemistry
- biological and chemical oceanography, biogeochemical cycling
- aquatic toxicology of silver and various silver compounds
- acute and chronic mechanism of toxicology of silver in fish
- water purification using colloidal silver
- vascular cell biology, with an interest in nanopatterned and nanostructured surfaces
- surface modification of polymers and metals at the nano level

Education

- Bioengineering
- Biologist (2 experts)
- Chemistry, polymer chemistry and biomaterials
- Civil and environmental engineering
- Earth science, geological and environmental sciences
- Toxicology
- Fish physiologist and toxicologist
- Molecular eco-toxicologist
- Neurotoxicology, metal and heavy metal toxicology
- Training not given

Bioavailability

Experts raised two concerns about bioavailability. One is specifically how nanosilver will enter and be transported within the body. This included understanding the cellular interactions between different cells and the nanosilver and the final fate of the particles. Factors such as surface interaction, accumulation and aggregation of the particles come into question. The second concern was the size of the particle, since this would not only determine the fate of the particles within the human body, but it would also allow the particles to behave uniquely in the environment. Nanoparticles within a watershed have the potential of transporting far greater distances than larger particles due to Brownian motion.

An initial study by Gao et al. (*37*) demonstrated that unfunctionalized nanosilver (20-40 nm) presents lower toxicity (or bioavailability), especially with increasing amounts of dissolved organic matter and increasing ionic strength.

Table 3. Exposure Risk Factors

Bioavailability - Could the nanosilver display bioavailability characteristics? (Bioavailability is the ability of a substance to be absorbed and used by the body. The more bioavailable it is, the greater is the chance for organisms to be exposed. This incorporates nanoparticle size as a factor to exposure.)
Rate of $\boldsymbol{Ag^+}$ ***release*** - Is the rate of silver ion release an important factor in evaluating exposure? (This factor is important because silver ions are considered one of the most toxic forms of silver.)
Rate of agglomeration - Is the rate of particle agglomeration (from “nano” to “not nano”) an important factor in evaluating exposure?
Coating/Matrix stability - Are there scenarios where the particle is freed from the coating/matrix?
Multiple disposal pathways - Does the product disposed in different ways contribute to different patterns of exposure, each with a different effect on the environment? This factor focuses on the issue of disposal/end of life treatment of a product.
Exposure route dependent - Does the route of a product during its life cycle help determine its exposure? (Can environmental factors such as water quality or soil content effect the overall exposure to nanosilver? Silver ions are highly reactive and will bind quickly with sulfur groups, especially in organic matter, minimizing their ability to cause harm, however it is unclear as to whether the same applies with these new applications of silver nanotechnology.)

Rate of Ag^+ *Release*

Rate of silver ion release is a critical factor in investigating nanosilver exposure as identified in the 0-hypothesis. One significant issue that relates to bioavailability was the question of whether exposure occurs to the nanoparticle or to silver ions being released from the particle. Since silver ions are highly reactive, they will be consumed upon release to the environment. On the other hand, the fate and transport of the nanoparticle is less certain (*38*). If the particle persists in the environment and continues to release silver ions, there is cause for concern, as the silver ion is considered the most toxic form of silver. Different environmental conditions cause particles to release varying levels of silver ions, resulting in more or less exposure to silver ions. Liu et al. (*39*) explored how factors like pH, dissolved oxygen level, temperature, water type (fresh vs. salt) affected ion release. Lowering pH, increasing dissolved oxygen, increasing temperature, and decreasing salinity were all significant factors to be considered independent from one another to explain the increased ion release. One dangerous exposure scenario, which demonstrates the problem with rate of silver ion release is accumulation of nanosilver within an organism. If conditions are such that the organism accumulates nanosilver within its internal tissues, then this nanosilver can release silver ions over time. There is hence, the potential for chronic exposure to a known toxic form of silver.

Rate of Agglomeration

The rate of agglomeration of silver nanoparticles was a more contentious issues amongst the experts. A majority of the experts (6 out of 10) were of the opinion that upon nanoparticle agglomeration, they are rendered nontoxic from a hazard viewpoint and are less bioavailable from an exposure viewpoint. The biologist and the molecular ecotoxicologist, however, were less certain, given uncertainities associated with solubility of these new agglomerated particles in various media. While agglomeration may change the toxicity potential of the particles, it is still an important factor when examining exposure to nanosilver.

Coating/Matrix Stability

Similar concerns exist for the coating/matrix stability criteria as for rate of agglomeration. How does a coating change the reactivity of the silver nanoparticle? Coatings, while meant to provide a way to affix nanosilver to certain products, can also allow for the antibacterial action of the silver to work without interference. One of the main concerns raised by the experts was the question as to how the nanosilver was adhered to the product: whether it was free or fixed. In some cases there was concern for the migration of nanoparticles from the coating into the surrounding environment. The major concern was for those applications where free nanosilver was coated on products and not secured by a matrix such as a polymer. This has been demonstrated to be a problem with textile coatings. In a recent study, Benn and Westerhoff showed that many athletic socks, treated with nanosilver, lost most of the total silver within the first few washes, suggesting that there should be concern about coating/stability with nanosilver products (*40*).

Multiple Disposal Pathways

Disposal of nanosilver can end in terminal points in water and or land. It was generally agreed on by 6 of the 10 experts that the majority of free nanosilver would end up in sewage treatment centers which, as the industry expert pointed out, would be predominantly removed in biosolids. This would place the burden to remove silver on treatment plants. However, these biosolids are frequently used as fertilizers for crops. The geochemist suggested this could result in creating problems for beneficial soil bacteria. Recently, Neal (*8*) postulated that nanosilver could affect many important bacterial populations such as soil and planktonic. Besides the use of biosolids containing nanosilver on crops, exposure to land did not emerge as a significant concern.

In Biotechnology and Nanotechnology Risk Assessment: Minding and Managing the Potential Threats around Us; Ripp, S., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2011.

Exposure Route Dependence

Exposure to nanosilver could change due to its surrounding micro-environment such as water characteristics or cell surface and charge interactions. Factors such as water characteristics have been shown to effect the exposure to metal ions. Two separate experts suggested a model based on EPA's regulation of copper in freshwater through the Biotic Ligand Model (BLM), where the amount of copper allowed is determined through a calculation that considers 10 different water criteria: temperature, pH, dissolved organic carbon (DOC), calcium, magnesium, sodium, potassium, sulfate, chloride, and alkalinity (*41*).

Table 4. Hazard Risk Factors: Material Properties

Particle size <100 nm* - Does the particle size factor into the hazard of the nanosilver application? (Nanotechnology is generally considered arbitrarily from 1-100 nm. The experts were asked how size played a part in the hazard of nanosilver.) *contained within the bioavailability factor in exposure related Risk Triggers.
Particle shape - Does the particle shape factor into the hazard of the nanosilver application? (Does the nanosilver display different reactivity characteristics due to shape?)
Aggregated nanoparticles - Are the properties of the free silver nanoparticles different from the aggregated forms of the nanoparticle?

Particle Size and Shape

Particle size and shape are the first two properties that the experts wanted to know about. Both size and shape help to determine where the nanosilver can migrate to and with what it can interact. In the SNCI, 45% of the 240 products reported the nanoparticle size used. They ranged from 0.3 nm to 250 nm with an average size of 24 nm (*33*). The expert in biomaterials noted that the most effective bactericides were those silver particles less than 10 nm. Cellular uptake was suggested to be higher for non-spherical shapes, however, spherical particles of sizes below 10 nm were considered to be just as significant by the bioengineer. Pal et al. (*11*) compared the antibacterial activity of different shape silver nanoparticles. They showed a difference in activity between nanoplates and spherical and rod-shaped nanoparticles and found nanoplates to be more effective at disinfection. Panacek et al. (*42*) demonstrated that the antibacterial activity was dependent on the size of silver particles. These studies support that the hazard associated with nanosilver can be both a function of size and shape.

Aggregated Nanoparticles

How does the aggregation of nanoparticles change their characteristics and hazard? As briefly discussed in the rate of agglomeration, there is a controversy on whether silver is rendered nontoxic when it is agglomerated or aggregated. It was suggested that one possible outcome of aggregated nanoparticles is that they start to display the same characteristic as those found in bulk silver. Ahamed et al. (*43*) postulated that coated particles were more effective at causing cell death due to the lack of agglomeration, suggesting that aggregated particles are less toxic than dispersed particles. However, Soto et al. (*44*) showed that aggregated nanomaterials such as silver can have cytotoxic effects on murine lung macrophage cell lines.

Table 5. Hazard Risk Factors: Material Reactivity

Catalytic action - Is catalytic action a factor in nanosilver antibacterial action? (In some cases it is thought that silver reacts with oxygen to produce antimicrobial activated oxygen or reactive oxygen species.)
Ag^+ release - What is the hazard of nanosilver releasing silver ions in the environment? (Previously, we looked at the exposure side of these particles releasing silver ions, while here we consider the associated hazard due to release. While low levels of Ag^+ on their own are not considered a large threat in the environment (due to their fast reactivity), do silver nanoparticles pose an environmental hazard through sustained Ag^+ release?)

All ten experts agreed that catalytic action and silver ion release were of the utmost concern in evaluating the hazard of nanosilver. The experts discussed the antibacterial properties of silver nanotechnology. Many of them referred to the concentration of bio-available silver as the important criterion, whether it be zero valent silver, silver nanoparticles (coated or functionalized), or silver ions. Some experts pointed out that there were still many data gaps which existed around understanding the antibacterial action of silver. The mechanism of antibacterial action of nanosilver is still poorly characterized. It is clear that silver ions that are released are toxic, but there is still a debate as to the influence of reactive oxygen species or direct particle interactions to the disinfection rates. Navarro et al. (*45*) and Kittler et al. (*46*) both explored the toxicity of nanosilver due to the silver ion. Navarro et al. summarized that the ionic silver measured in the silver colloids could not fully explain the observed mechanism, suggesting that other antimicrobial mechanisms might play a significant role in disinfection (*45*, *46*). Toxicology studies by Choi et al. (*47*), Carlson et al. (*48*), Hsin et al. (*49*), and Roh et al. (*50*) have pointed to the role of oxidative stress as being a primary mechanism for nanosilver toxicity. Nanosilver has also been shown to affect mammalian cells by binding to proteins and enzymes. This causes co-localization of the cell wall and the toxic reactive oxygen species can cause inflammation and destroy cellular elements like mitochondria resulting in cell death (*51*). On the other hand, nanosilver has been shown to be beneficial in medicine, by promoting the healing of wounds (*52*).

Table 6. Hazard Risk Factors: Material Toxicity

Dermal toxicity - Hazard level due to dermal toxicity. (How dangerous is nanosilver in dermal contact?)
Toxicity in lung - Hazard level of nanosilver through entry into the lung. (How dangerous is nanosilver if absorbed in the lung or gill?)
Toxicity from chronic exposure - Hazard level for chronic exposure to nanosilver. (How dangerous is it to be chronically exposed to nanosilver, even at a low dose?)
Toxicity from ingestion - Hazard level for ingestion of nanosilver. (How dangerous is nanosilver if ingested, either through diet or another means?)
Toxicity in Salt Water - Hazard level of nanosilver in a marine environment. (How dangerous is nanosilver in salt water? Why is it less or more toxic than in fresh water?)
Toxicity in Fresh Water - Hazard level of nanosilver in a freshwater environment. (How dangerous is nanosilver in fresh water? Why is it less or more toxic than in salt water?)

Table 7. Exposure Scenarios

Absorption (dermal) - The level of risk of exposure to nanosilver through dermal absorption.
Inhalation/Absorption (lung) - The level of risk of exposure to nanosilver through inhalation or absorption through the lung.
Ingestion - The level of risk of exposure to nanosilver through ingestion. This can include ingestion from a food source or from the use of the product.
Aquatic release exposure - The level of risk of exposure that nanosilver is released into the aquatic environment.
Nanosilver migration through the food chain - The level of risk that nanosilver will migrate though the food chain through bioaccumulation across species.
Chronic exposure - The level of risk of chronic exposure to nanosilver. This includes the potential for chronic exposure in the environment and the risk of exposure from multiple consumer products.

Ranked Risk Factors

The ranking of hazard factors (material toxicity) and exposure scenarios on a scale of 1 to 5 (5 having the most risk) based on coded interview transcripts from the expert elicitation is presented in Figure 2. Not all experts had opinions about each factor or scenario. The number of experts to comment on a particular factor or scenario is listed in parentheses. Hazard factors of high risk included toxicity in freshwater, in lung and from chronic exposure. High risk exposure scenarios mirrored that of the hazard factors, placing aquatic release as being of primary concern, followed by chronic exposure and dermal absorption.

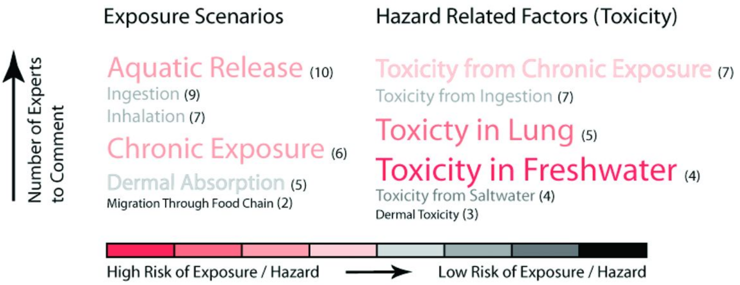

Exposure Scenarios
Hazard Related Factors (Toxicity)
Number of Experts to Comment
Aquatic Release (10)
Ingestion (9)
Inhalation (7)
Chronic Exposure (6)
Dermal Absorption (5)
Migration Through Food Chain (2)
Toxicity from Chronic Exposure (7)
Toxicity from Ingestion (7)
Toxicty in Lung (5)
Toxicity in Freshwater (4)
Toxicity from Saltwater (4)
Dermal Toxicity (3)
High Risk of Exposure / Hazard
Low Risk of Exposure / Hazard

Figure 2. Ranked Exposure Scenarios and Hazard Related Factors. The above figure displays the relative risk of exposure or hazard of each exposure scenario and toxicity hazard related factors. Font that is larger and in warmer colors indicates high risk while small font and cooler colors indicate low risk. The number of participating experts on each topic is noted next to the factor. (source: JMLE article)

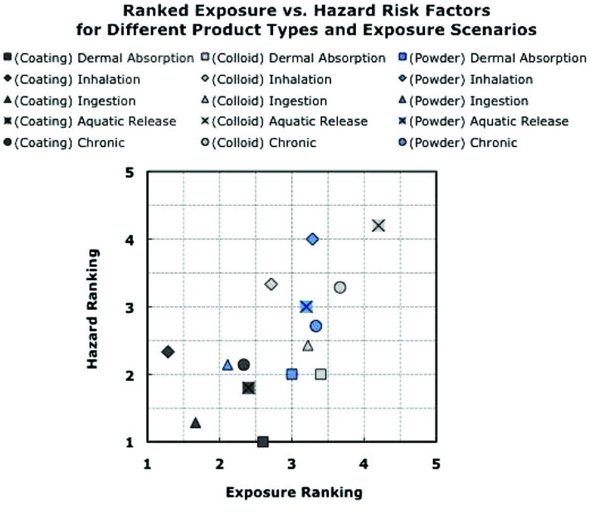


Figure 3. Ranked Exposure vs. Hazard Risk Factors Matrix by Product Type. The average ranking of each factor, based on the expert interviews, on a scale of 1 to 5, with five representing high risk and one representing low risk is presented. Exposure scenarios were paired with their corresponding hazard factor for a comparison of risk between the factors for different product types.

Figure 3 shows a risk matrix of hazard factor (material toxicity) vs. exposure scenario rankings by product type (coating, colloid or powder). Expert scores inferred from the transcribed interviews were tallied for each factor and scenario. Scores were adjusted according to product categories: coating, colloid or powder and based on statements made by the expert. This analysis of the interview results shows that out of the 10 experts interviewed, the primary areas of high risk are colloidal based nanosilver products gaining exposure to the aquatic environment or having chronic exposure to colloidal silver. This includes health effects to both aquatic organisms and humans.To compare our analysis with other nanotechnology risk assessment results, we present in Table 8 a list of physicochemical characteristics that were determined to be important in the characterization of nanometals by the EPA in 2009. Many of these are not required for registered pesticide ingredients. Most of these are related to or are included within the exposure and hazard factors identified in this study.

Table 8. Physicochemical Characteristics Important in Characterizing Nanometals

1. Physical state
2. Chemical composition
3. Solubility
4. Density
5. Cation/Anion exhange capacity*
6. Size and distribution of nanoparticles
7. Surface area (m^2/g); surface reactivity*
8. Zeta Potential*
9. Surface charge*
10. Catalytic properties*
11. Aggregation/agglomeration, conditions under which they occur (pH, temperature, etc.)*

"Information about properties 5-11 (*) is not routinely required for pesticide ingredients, but EPA believes they would be helpful for the characterization of nanometals" (*20*).

Exposure and Hazard Product Maps for High Risk Areas

To most effectively use this type of analysis in adaptive management and risk assessment, the data from expert elicitation must be presented in the context of the experts' reasoning, product information and information from current literature. This is necessary so that the details captured in the exposure and hazard factors contained within a product map that includes elements like the product lifecycle. For instance, considering the highly ranked risk scenarios of aquatic and chronic exposure to colloidal silver, we constructed a product map to identify the stages in the product lifecycle where these risks may occur.

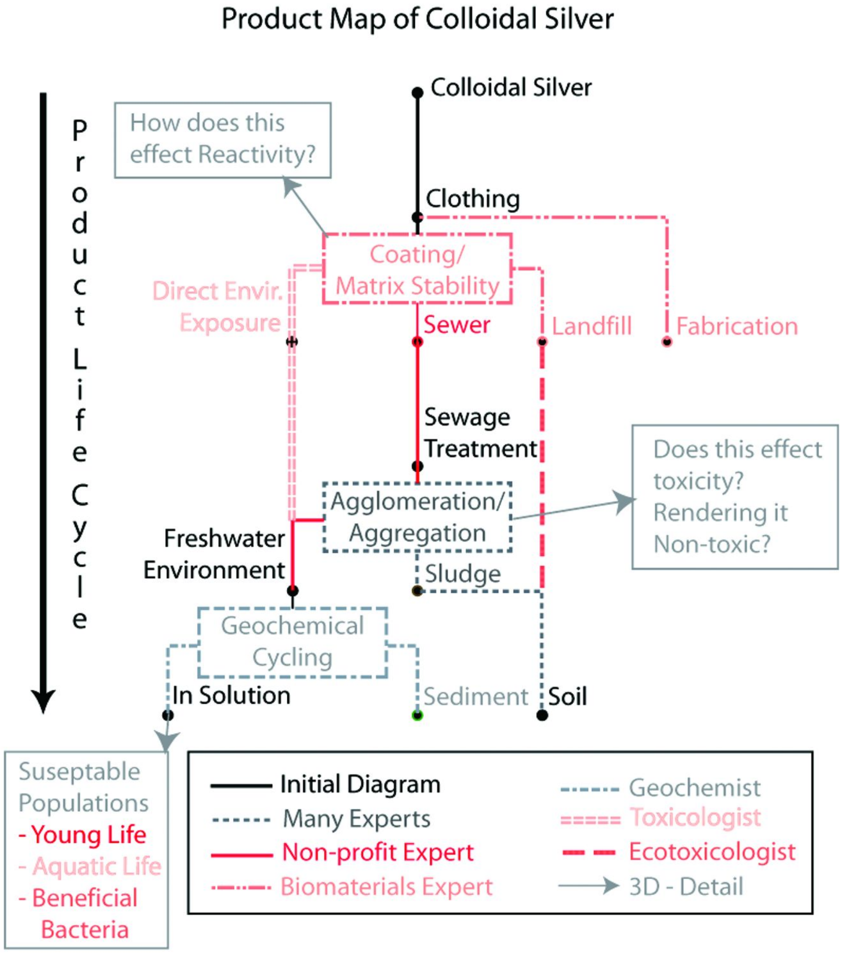

Product Map of Colloidal Silver
Product Life Cycle
Colloidal Silver
How does this effect Reactivity?
Clothing
Coating/ Matrix Stability
Direct Envir. Exposure
Sewer
Landfill
Fabrication
Sewage Treatment
Does this effect toxicity? Rendering it Non-toxic?
Agglomeration/ Aggregation
Freshwater Environment
Sludge
Geochemical Cycling
In Solution
Sediment
Soil
Suseptable Populations
- Young Life
- Aquatic Life
- Beneficial Bacteria
Initial Diagram
Many Experts
Non-profit Expert
Biomaterials Expert
Geochemist
Toxicologist
Ecotoxicologist
3D - Detail

Figure 4. Product Map of Colloidal Silver. Above is an excerpt of the product map of colloidal silver derived from the expert elicitation process. Starting at the initial product of colloidal silver, its lifecycle is traced down the path of being incorporated into clothing. This demonstrates the path of the nanosilver, potential endpoints of nanosilver and decision criterion that influence whether nanosilver goes down one path or another. The graph is color coded to show how different experts contributed to different information. The 3D – detail boxes illustrates how there are more layers of details uncovered by the interviews that are important in evaluating risks.

Of all the nanosilver products on the market, the experts judged that the largest chance of exposure came from colloidal nanosilver products. Products like cleaning sprays and clothes that require washing, raised concerns for exposure. Beneficial bacterial populations that are used to treat sewage could be directly effected by nanosilver. The work of Choi et al. (*53*) shows that nitrifying bacteria are especially susceptible to inhibition by nanosilver and suggest that nanosilver could have detrimental effects on microorganisms in wastewater treatment (*53*). Asharani et al. (*54*), Lee et al. (*55*), Lubick et al. (*56*), and Navarro et al. (*57*) have shown nanosilver to be toxic to various other aquatic organisms. Coatings and particle size could increase the duration of exposure by preventing agglomeration and allowing transport of these particles to locations that were previously unavailable to silver ions due to their limited lifetime. Figure 4 demonstrates the path and decision criteria that are important in evaluating nanosilver in a product such as clothing.

Chronic exposure could lead to accumulation of silver nanoparticles in areas such as the liver, spleen and interstitial tissue. In a 28 day oral toxicity rat study on silver nanoparticles, Kim et al. (*58*) observed a dose dependent accumulation of nanosilver in the kidneys. In situations where nanosilver does not reach acute levels in the aquatic environment, the experts expressed concern about the chronic effects of nanosilver, especially in susceptible younger developing aquatic organisms. Naddy et al. (*59*) studied toxicity in fathead minnows and concluded that the mechanism of chronic silver toxicity may be the same as that for acute toxicity. It has been shown in long term studies that most of the silver deposits in the liver and induces the production of a certain protein (*60*). It has been suggested that the production of this protein might be detrimental to the animal. In addition to these possible detrimental effects, widespread use of chronic low-level doses of silver as an antimicrobial increases the chances of bacterial resistance.

Another area of primary concern is inhalation of products that contain nanosilver powder or colloids, such as in some athletic socks or cleaning sprays. AshaRani et al. (*61*) showed that human lung fibroblasts and glioblastoma cells, when exposed to silver nanoparticles exhibited dose dependent damage to DNA and mitochondria while ATP content was reduced and reactive oxygen species production was increased. Sung et al. (*62*), in a 13 week rat study, showed a dose-dependent increase in lesions related to silver nanoparticle exposure, including mixed inflammatory cell infiltrate, chronic alveolar inflammation, and small granulomattous lesions. Inhalation of colloids for aquatic organisms becomes a significant issue because nanoparticles can interfere with the lungs, acting as an irritant, which leads to increased mucus production and poorer oxygen uptake. Silver in fresh water has been shown to block the active uptake of sodium in the gills and thereby cause ion regulatory failure resulting in cardiac arrest. Areas of low risk included many of the coating based products. The leaching or residue from products coated with nanosilver were judged to pose an overall lower risk in terms of exposure and hazard. In addition to the identified risks, the expert elicitation process helped to identify specific knowledge gaps (Table 9) and susceptible populations (Table 10) that might be more sensitive to exposure to nanosilver. These are factors that should be considered in any systems approach for managing and exploring silver nanotechnology.

Table 9. Knowledge Gaps

Toxicity • Basic toxicity testing on silver nanoparticles • Investigate human health risk specifically to nanosilver and its long term effects • Long and short term studies on the effects of nanosilver on organisms from different taxa • Silver nanoparticle effects on cells
Transport • Life cycle analysis • Research on transport mechanisms • Geochemical cycling analysis of silver nanoparticles
Particle Properties/Mechanisms • Research on stability of silver nanoparticles • Mechanisms of antibacterial actions (ion release and reactive oxygen species) • Investigate risk posed by agglomerated particles

Table 10. Susceptible Populations

Fish
Organism at the bottom of the food chain
Early life stages
Beneficial bacteria

Acknowledgments

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Chapter 3

Environmental Application and Risks of Nanotechnology: A Balanced View

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As a promising "green" technology, nanotechnology has great potential for improving environmental quality, reducing consumption of resources and energy, and allowing environmentally benign economic development. However, rapid development and expanding use and disposal of nano-products very likely poses complex risks to the environment and biological systems due to the tiny size and high surface area of nanoparticles. This chapter provides a brief review on the current and potential applications and hazards of nanotechnology to the bio-environmental systems. It is suggested that environmental safety regulations must ensure that benefits significantly outweigh the risks of each new nanotechnology before it is used on a large scale. For this, a multi-dimensional life cycle analysis that involves both biotic and abiotic systems should be performed to assess the long-term impacts of nanoparticles in terms of their type, structure, size, and application. Uncertainty associated with life-cycle risk assessment must be clarified. A social alerting system should be established to make consumers aware of nanomaterial-containing products, their potential hazards, and the best disposal methods (e.g., via barcode labeling). Nanotechnology may represent one of the most profound impacts of humans on nature in the long term. Therefore,

In Biotechnology and Nanotechnology Risk Assessment: Minding and Managing the Potential Threats around Us; Ripp, S., et al.;
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nanotechnology development must be driven by long-term benefits and strict risk mitigation policy on a global scale.

1. Introduction

Nanotechnology, which involves materials and processes on an ultra-small scale, has the potential to affect environmental quality and ecological services through nano-product production, site remediation, and waste treatment. These effects can be both positive and negative. On the positive side, nanotechnology can be used to treat drinking water, eliminate toxic chemicals, reduce water and energy consumption, and harness clean energy technologies. However, the burgeoning nanotechnology industry is flooding the marketplace with nanotechnology products that have potential to release large amounts of nanowaste to the soil, water, and air (*1*, *2*). These nanowastes may pose risks to the environment and human health in ways that their microscale counterparts do not (*3–9*).

The greatest potential uses or applications of nanotechnology for the environment include waste treatment, site remediation, and sensors (*10*). These applications can be categorized as either reactive to existing environmental problems or proactive in anticipating and preventing future problems. The former includes sorbing and decomposing organic contaminants in the environment using nanomaterials (such as nanoscale photocatalysts, nanoscale zerovalent iron, and polymeric nanoparticles) and detecting chemical and biological agents using nanosensors. The latter includes nanofiltration, nanocomposites for removing metals from smokestack emissions, and green manufacturing.

While nanotechnology can provide solutions for certain environmental problems, there may be hazards to the environment and biological systems due to the tiny size and high surface area of nanoparticles, which make them more chemically reactive and causes them to behave in unpredictable ways (*11–14*). A substance that is safe at microscale size can become toxic at the nanoscale because the nanoscale size and high surface area make it more soluble and easy to uptake. The issues of concern include toxicology, bioaccumulation, and biomagnification. Little is known about the biological and environmental fate, transport, persistence, and transformation of nanomaterials or of the resulting broader ecological impacts. Potential negative impacts may be due to toxic nanoparticles being released to the environment or from the release of environmentally damaging synthetic chemicals used to produce nanoparticles (*2*, *15*). To understand the potential risks, life cycle analysis is needed to assess the long-term impacts of nanoparticles in terms of their type, structure, size, and application (*11*, *16*).

This chapter posits that, while nanotechnology offers significant opportunities for improving the environment, further research on the exposure routes and potential risks is required in order to achieve safe, healthy, and sustainable application.

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2. Life Cycle Impacts of Nanomaterials

Assessment of the toxicology and exposure of manufactured nanomaterials in the environment requires a life cycle analysis (LCA). LCA tracks a nano-product from its inception through its final disposal. Using LCA can avoid problem-shifting, where an improvement at one stage of life cycle causes new problems at another stage. LCA can not only help us understand the overall health and environmental impacts of nano-products, but can also support the development of decisions on the production and management of nanomaterials (*16*). The LCA can answer a series of questions such as:

- What are the impacts of nanomaterials?
- How do risks change throughout the stages of the life cycle of nano-products?
- What are the trade-offs between potentially negative eco-toxicological impacts and potential environmental gains?

A simplified framework of the life cycle stages of nanomaterial products and associated benefits and hazards is presented in Figure 1. The life cycle of nano-products involves input risks associated with consumption of energy and materials and potential output risks associated with pollution/damages to bioenvironmental systems. Assessment of the total environmental impacts of a nano-product requires analysis of the inputs and outputs in each life-cycle stage. The focus of the analysis should be the degrees of exposure and responses of bioenvironmental systems to the hazards. However, development of effective monitoring methods or tools for nanomaterials is still a challenge (*17*). New approaches are also required for information integration and uncertainty assessment (*13*).

3. Environmental Applications

Nanotechnology has great potential to remediate various environmental problems as well as to monitor environmental pollutants (*18–25*). Nanotechnology can help develop new, environmentally safe, and green technologies that minimize the formation of undesirable by-products or effluents. Nanotechnology is already being used to improve water quality and to assist in environmental clean-up activities. Their potential use as environmental sensors to monitor pollutants is also becoming viable (*26*). The following topical summaries show a broad range of application and capability of nanotechnology in improving the environment.

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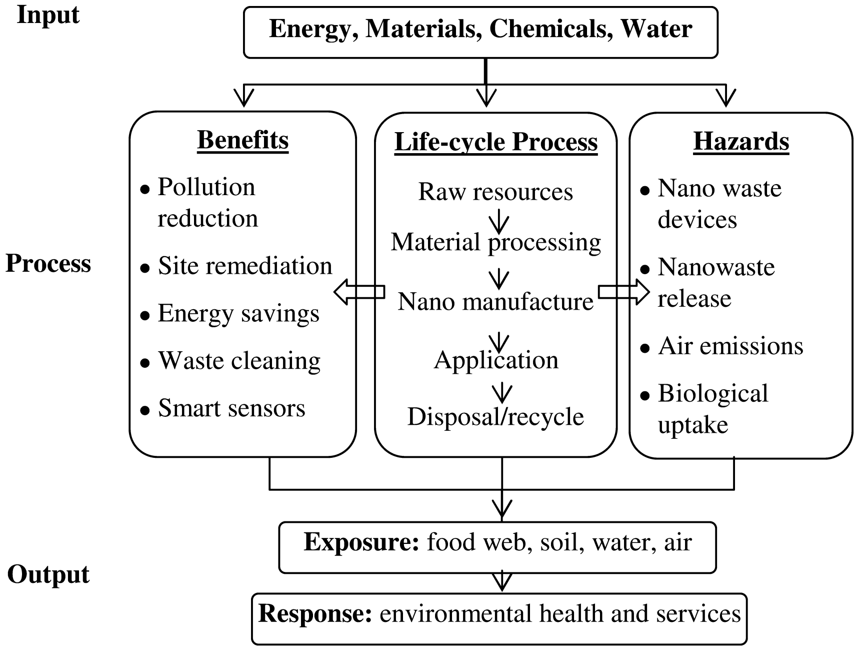
Input
Energy, Materials, Chemicals, Water
Process
Benefits
• Pollution reduction
• Site remediation
• Energy savings
• Waste cleaning
• Smart sensors
Life-cycle Process
Raw resources
Material processing
Nano manufacture
Application
Disposal/recycle
Hazards
• Nano waste devices
• Nanowaste release
• Air emissions
• Biological uptake
Output
Exposure: food web, soil, water, air
Response: environmental health and services

Figure 1. Environmental benefits and hazards associated with the life-cycle process of nanomaterials.

3.1. Subsurface Remediation − A Nanoiron Example

Remediation is one of the reactive applications of nanotechnology. Several types of nanomaterials have been considered for remediation purposes, such as nanoscale zeolites, metal oxides, and carbon nanotubes and fibers (*12*). Nanoscale particles used in remediation have the ability to access areas that larger particles cannot, and they can be coated to facilitate transport and prevent reaction with surrounding soil matrices before reacting with contaminants. One of the widely used nanomaterials for remediation is nanoscale zerovalent iron (nZVI). It has been used at a number of hazardous waste sites to clean up chlorinated solvents that have contaminated groundwater. Specifically, nZVI is introduced in the groundwater to catalyze the removal of chlorine molecules from common solvents such as perchloroethylene and trichloroethylene (PCE and TCE). The dechlorination of these substances eventually breaks down the PCE and TCE to carbon dioxide. The reaction rates of nZVI are 25–30 times faster than the reaction rates of microsized granular iron (*21*). Studies indicate that more TCE is reduced as the sizes of nZVI particles get smaller. Iron particles with a diameter of two micrometers can reduce 0.186 mg TCE per mg Fe per hour, but this rate increases to 186 mg TCE per mg Fe per hour when the iron particles are as small as two nanometers (*27*). Overall, the nanosized particle is three orders of magnitude smaller, but its rate of TCE reduction is three orders of magnitude greater than that of the microsized particle. In addition, nZVI has been found to immobilize arsenic, chromium, and lead, which are three highly toxic metals used in industry and common at hazardous waste sites. Nanosized iron oxide could bind arsenic irreversibly up to 10 times more effectively than microsized particles, resulting in removal of ~99% of arsenic (*11*). Kanel et al. (*23*) reported that nZVI can reduce As(V) to As(III) in a short period of time at neutral pH.

The efficient use of nanotechnology in site clean-up is limited by the fate and transport of nanoparticles. It is imperative that nanoparticles reach the endpoint of the remediation cycle. However, nanoparticles easily aggregate and react with environmental media, particularly at high concentrations. As a result, the nanoparticles get larger (even larger than 100 nm) after they enter the subsurface environment. The aggregation, plus changes in surface properties after reaction with contaminants and attachment on environmental media, can shorten the travel distance of the nanoparticles in soil and groundwater and eventually weaken their reactivity. For example, the effectiveness of nZVI diminishes as particles age and, as a result, their reactivity only lasts for approximately one year in groundwater under certain conditions (*22*). Interactions with natural organic matter and organisms (e.g., plants, algae, and fungi) also affect the availability and accessibility of nanoparticles and their effectiveness in remediation (*28*). Therefore, scientists are seeking ways to enhance the mobility of reactive nanoparticles (e.g., nZVI) so that they remain nanoparticles for a long time and thus travel further from the point of origin to clean up more pollution. One of the approaches is to encapsulate the nanoparticles (as used for nZVI) in a protein shell to facilitate transport and reaction with pollutants (e.g., hexavalent chromium) in groundwater systems.

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3.2. Water Cleanup

Nanotechnology can facilitate the development of new purification schemes to improve current technologies such as new membranes (e.g., containing self-assembling photoactive pores) for nanofiltration or nanoseparation, reverse osmosis, sorption, and photocatalysis (*24*). For example, nanoparticles of cerium oxide (CeO_2) can significantly adsorb chromium (IV) from water (*29*). TiO_2 nanoparticles, or their modified versions, are highly efficient photo-oxidants for removing organic pollutants from water (*30*). TiO_2 can even be functionalized with organic molecules (e.g., ethylenediamine) to remove anionic metals from groundwater (*31*). Carbon nanotubes (CNTs) are generally 0.4-2.5 nm in size for single walled CNTs (SWCNTs) and up to several hundred nanometers for multi-walled CNTs (MWCNTs). They have been found to be effective for a variety of organic compounds (e.g., DDT, pesticides, phenols, and PAHs) (*25, 32–36*). CNTs can be arranged to form a hollow monolithic cylindrical membrane (*37*) that is efficient for the removal of bacteria or hydrocarbons and can easily be regenerated by ultrasonication or autoclaving. Nanofiltration membranes are pressure-driven membranes with pore sizes between 0.2 and 4 nm. Incorporation of surface-derivatized nanoparticles could make the membranes highly efficient in the removal of dissolved organic matter and trace pollutants from water.

3.3. Carbon Capture

Carbon dioxide is considered to be one of the major greenhouse gases directly influencing global climate change (*38*). It is estimated that over one-third of the United States' anthropogenic CO_2 is produced from coal-fired power plants (*39*). Consequently, the capture and sequestration of CO_2 from flue gas streams is a critical step for carbon management in the environment. Several approaches (e.g., solvents, cryogenic techniques, membranes, and solid sorbents) have been investigated for CO_2 capture. Among them, aqueous amine solutions have been widely used, but they present a corrosion problem for equipment and they degrade through oxidation. Solid sorbents offer a number of advantages, including low energy requirements for sorbent regeneration and elimination of corrosion problems (*40*). Amine grafted solid sorbents have thus shown great promise. However, the amount of amines that can be immobilized is still relatively small. The development of an economic CO_2 separation process requires a highly efficient CO_2 sorbent. The sorbent must possess high CO_2-capture capacity, long-term use, and the ability to be regenerated with a small difference in adsorption and desorption temperatures. Use of nanomaterials is a potential approach to preparing such a sorbent. They have a high surface area for immobilizing amine compounds and possibly other polymers as much as 100 times more than currently studied solid sorbents (*41*). Electrostatic layer-by-layer self-assembly (LBL) is an approach that is based on sequential absorption of polymers with inorganic nanocrystals (*42*). LBL is the most promising method for preparation of stable multi-layer nanocoatings of controlled thickness and molecular architecture (e.g., roughness) (*43*). Li et al. (*44*) used LBL nanoassembly to build nano-layers of CO_2-adsorbing polymers (e.g., polyethylenimine) within porous particles serving as sorbents for CO_2 removal. The nano-layers are developed by alternately immobilizing a CO_2-adsorbing polymer and an oppositely charged polyelectrolyte (e.g., polystyrene sulfonate) on porous particles (e.g., polymethylmethacrylate). The layers have fast CO_2 adsorption (within seconds) and desorption properties (within ~30 minutes for 90% adsorbed CO_2) and their CO_2 capture capacity increases with the number of nano-layers of the CO_2-adsorbing polymer (Figure 2). Overall, the LBL process is simple and environmentally friendly. It can be operated at room temperature in a pre-designed order on any shape of substrate that is favorable to fast CO_2 transport (*44*).

Trapping CO_2 in nanopores based on a metal-organic framework (MOF) is another innovative approach that is in rapid development (*45*, *46*). MOFs represent a class of porous materials that offer the following advantages for CO_2 storage: ordered structures, high thermal stability, adjustable chemical functionality, extra-high porosity, and the availability of hundreds of crystallines (*47*) (Figure 3). Recently, Bureau De Recherches Geologiques Et Minieres (BRGM) inventors developed a low-cost method for CO_2 capture using nanomaterials (such as mesoporous silica and carbon nanotubes) in a United States Patent Application (*48*). The method enables CO_2 trapping in a reversible manner under conditions of near ambient pressure (0.2-3 bars) and temperature (0-30°C). The process does not need any handling of the suspension constituting the trap, which remains

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in place in the capture/release reactor throughout the cycle. The capture and release of CO_2 are controlled by changing pressure (or partial pressure of CO_2) and temperature of the system. A French research team, led by Gérard Férey at the University of Versailles, has developed a new porous nanomaterial, MIL-101 (also known as chromium terephthalate); MIL stands for Matériaux de l'Institut Lavoisier. It is used to sequestrate CO_2 released from power plants, tailpipes, and smokestacks (*49*). MIL-101 has a zeotype cubic structure with a giant cell volume (~702 nm^3), a hierarchy of extra-large pore sizes (3.0-3.4 nm), and a N_2–based Langmuir internal surface area of 5,900 ± 300 m^2/g. Later, Chowdhury et al. (*50*) and Zhang et al. (*51*) examined the adsorption isotherma of CO_2 on MIL-101 at different temperatures and pressures. Hong et al. (*52*) further examined the site-selective functionalization of MIL-101 and demonstrated that surface amine-grafting could provide a general way of making MIL-101 a size-selective molecular sieve catalyst. Chen and Jiang (*53*) investigated a recently synthesized bio-metal-organic framework (bio-MOF-11) for CO_2 capture by molecular simulation. Bio-MOF-11 exhibits larger adsorption capacities compared to numerous zeolites, activated carbons, and MOFs. The difference is attributed to the presence of multiple Lewis basic sites and nano-sized channels in bio-MOF-11. The results for the adsorption of CO_2/H_2 and CO_2/N_2 mixtures in bio-MOF-11 show that CO_2 is more dominantly adsorbed than H_2 and N_2, suggesting that bio-MOF-11 has significant potential for pre- and post-combustion CO_2 capture.

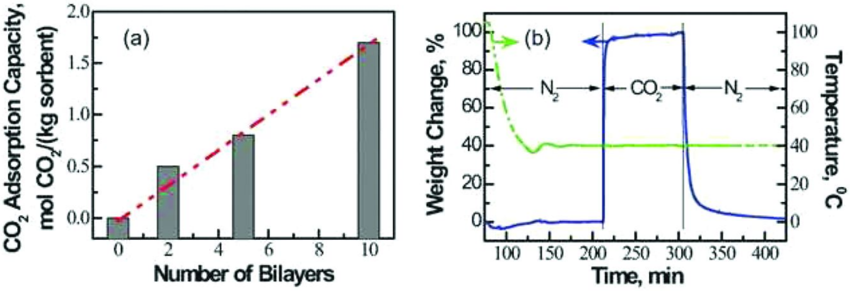

Figure 2. (a) CO_2 adsorption capacity vs. number of polyethylenimine/ polystyrene bilayers. (b) Typical CO_2 adsorption/desorption curves of polyethylenimine/polystyrene sulfonate nanolayered solid sorbents. Weight change (solid blue line), temperature (dashed green line). (Reproduced with permission from Li et al. (44). Copyright 2011 the Royal Society of Chemistry.)

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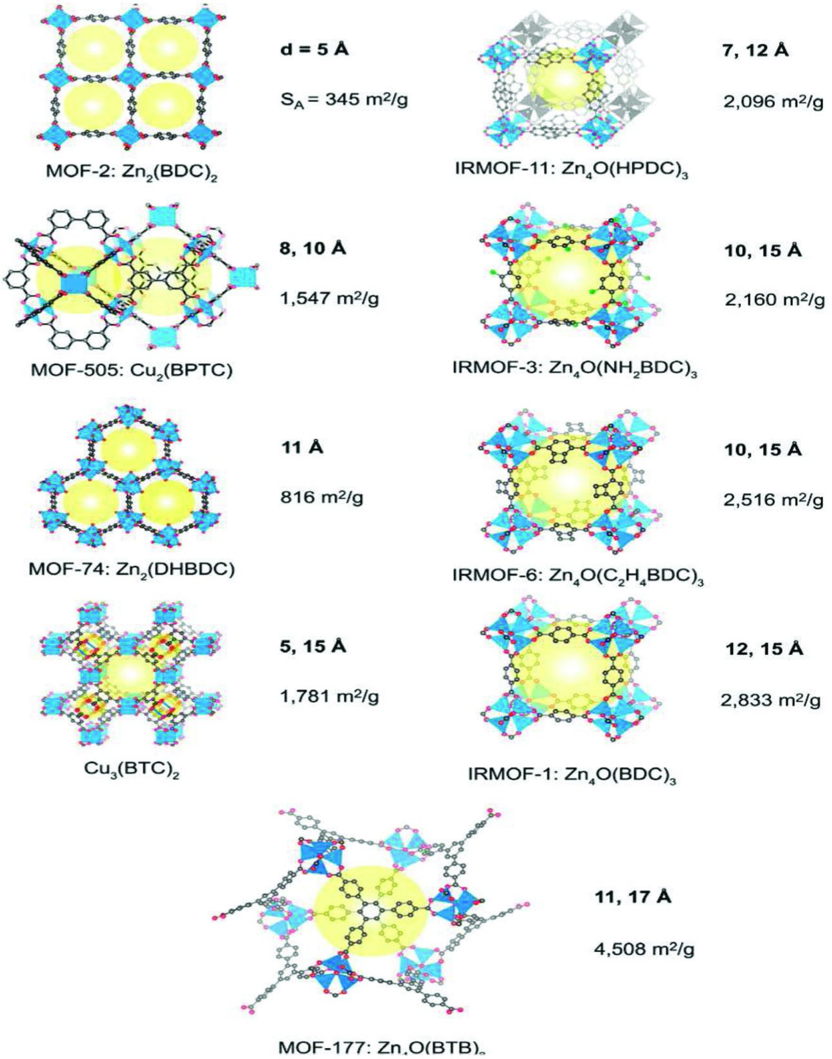
d = 5 Å
S_A = 345 m²/g
MOF-2: $Zn_2(BDC)_2$
7, 12 Å
2,096 m²/g
IRMOF-11: $Zn_4O(HPDC)_3$
8, 10 Å
1,547 m²/g
MOF-505: $Cu_2(BPTC)$
10, 15 Å
2,160 m²/g
IRMOF-3: $Zn_4O(NH_2BDC)_3$
11 Å
816 m²/g
MOF-74: $Zn_2(DHBDC)$
10, 15 Å
2,516 m²/g
IRMOF-6: $Zn_4O(C_2H_4BDC)_3$
5, 15 Å
1,781 m²/g
$Cu_3(BTC)_2$
12, 15 Å
2,833 m²/g
IRMOF-1: $Zn_4O(BDC)_3$
11, 17 Å
4,508 m²/g
MOF-177: $Zn_4O(BTB)_2$

Figure 3. Crystal structures of MOFs examined for CO_2 storage capacity at room temperature. For each MOF, the framework formula, pore size, and surface area are given. (Reproduced with permission from Millward and Yaghi (47). Copyright 2005 American Society of Chemistry.)

Nanotechnology may also have potential to play a role in carbon sequestration underground. A research team, led by Jeffrey Urban, at the Lawrence Berkeley National Laboratory (LBNL), recently produced nanoscale magnesium oxide crystals that can facilitate the CO_2-solid bonding process, in which CO_2 attaches to a solid and forms a carbonate. The process otherwise takes thousands of years under natural conditions. The LBNL scientists found that the size and surface chemistry of nanocrystals have significant effects on the rate of CO_2 capture (*54*).

The results suggest the possibility of an instant assessment on how much storage space is needed and how long it will take to store the CO_2 after injection.

Such promising results demonstrate that nanotechnology may lead the way in decarbonizing the economy by promoting renewable energy and developing strong adsorbents. Scientists may be able to create an array of three-dimensional synthetic nanocrystals that allow carbon dioxide to be captured much more effectively.

3.4. Pesticides and Fertilizers

Current agricultural technology requires extensive use of fertilizers to provide nutrient fixation to crops. Nutrient optimization is needed to avoid excess; nanoparticles for direct nitrogen fixation might prove revolutionary, or there may be opportunities to engineer soil for fertilization improvement. Nanocapsules have already been incorporated in the development and formulation of agrochemicals by providing a more efficient and controlled delivery/release system for the application of pesticides and fertilizers (*55*, *56*). Overall, nanotechnology can reduce use of pesticides, herbicides, and rodenticides and therefore lower undesirable impacts. Further, nanotechnology may add benefit by the development of photo-catalysts to remove biocides in the environment (*57*) and by the development of "smart dust" (tiny, wireless networks of sensors) to identify and locate biocides in the environment. For example, nanostructured alumina was found to be a cheap and reliable alternative for control of insect pests in stored food supplies (*58*). The study reported that two major insect pests (*Sitophilus oryzae* L. and *Rhyzopertha dominica* F.) experienced significant mortality after three days of continuous exposure to nano-alumina treated wheat. Establishment of a knowledge base that relates structure and function at the nanoscale and design of new materials and architectures with tailored multifunctionality will aid in the development of agricultural applications. However, nanomaterials may be taken up by plants and thus enter the food web. Therefore, caution must be taken if nanomaterials are used in the production of organic agro-products.

3.5. Environmental Sensing

Research has shown that nanotechnology might be able to provide more sensitive detection systems for monitoring water and air quality, allowing for accurate, real-time, simultaneous sensing of a variety of compounds at low concentrations and measurements of environmental parameters (*59*). The small size and wide detection range of the nano-sensors provide great flexibility in practical applications. It has been reported that nanoscale sensors can be used to detect microbial pathogens and biological compounds such as toxins in aqueous environments (*26*, *60*). For example, Wang et al. (*61*) developed an electrical sensor to measure microcystin-LR (MC-LR), which is one of the most common and the most dangerous toxins to mammals produced by cyanobacteria. The sensor was developed by impregnating common filtration papers with carbon nanotubes and antibodies. It shows linear response to MC-LR concentrations from 0.6 nmol/L to 10 nmol/L and nonlinear detection up to 40 nmol/L. Compared with

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the traditional best biochemical technique (i.e., enzyme-linked immunosorbent assay), this new approach reduces the analytical time by at least 28 times. If the antibody in the sensor electrode is changed, the sensor has potential to be modified for detecting many other harmful chemicals or toxins in water or food. Nanomaterials (mostly metal-based) can be used to improve the performance of gas sensors. They include single-component (e.g., ZnO, SnO_2, WO_3, TiO_2, and Fe_2O_3) (*62*, *63*) and multi-component oxides ($BiFeO_3$, $MgAl_2O_4$, $SrTiO_3$, and $Sr_{1-y}Ca_yFeO_{3-x}$) (*64*). The main mechanism for gas detection in these materials is based on the change in electrical conductivity resulting from electron transfer in the reactions between the oxygen of the sensor surface and the reactive gas. Another possible application of nanotechnology is to trace pollution attribution. By incorporating nanoparticles of signature chemical compositions into point and distributed emission or flow sources (e.g., power plants and groundwater systems), one can distinguish anthropogenic from natural nanoparticle pollutants. Overall, nanotechnology provides easy ways to monitor various pollutants at low cost as compared to conventional methods. However, further work is needed to improve repeatability and control of sensor systems.

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3.6. Air Purification and Emission Mitigation

Nanotechnology can be used to improve air quality. For instance, surface-doped titanium dioxide nanoparticles can clean air through catalysis under visible light (wavelength <500 nanometers) (*65*, *66*). TiO_2 shows the most efficient photocatalysis under ultraviolet (UV) light (wavelength <387 nm), which corresponds to 3-4% of the solar spectrum. Development of visible-light driven photocatalysis of TiO_2 will not only greatly increase the outdoor photocatalytic activity but also enable indoor applications where there is little UV light. Asahi et al. (*65*) found that nitrogen doping on titanium oxide ($TiO_{2-x}N_x$) increases light absorption and makes titanium oxide catalytically active under visible light. A number of studies have revealed that TiO_2 doped with non-metal atoms, such as carbon (*67*, *68*), fluorine (*69*, *70*), sulfur (*71*), bromide and chloride (*72*), and iodide (*73*), shifts the optical absorption edge of TiO_2 to a lower energy range, leading to an increase in photocatalytic activity under visible light. This enhanced photocatalysis allows the main part of the solar spectrum, and even the poor illumination of interior lighting, to be used to degrade gaseous organic pollutants in the air (e.g., benzene, acetaldehyde, and carbon monoxide). This principle of photocatalysis has been applied to commercial nano-products, such as self-cleaning glasses, architectural coatings, and building blocks. Some nanomaterials can even remove metal contaminants from air. For example, silica-titania nanocomposites can remove elemental mercury from vapors such as those generated from combustion sources, with silica serving to enhance adsorption and titania to photocatalytically oxidize elemental mercury to the less volatile mercuric oxide (*74*). Studies have also demonstrated that nanostructured silica can sorb lead and cadmium generated in combustion environments (*75*, *76*).

Nanotechnology can offer solutions to mitigating greenhouse gas emissions through three approaches: waste gas treatment, green manufacturing, and green energy development. Nanomaterials have potential for adsorbing and

In Biotechnology and Nanotechnology Risk Assessment: Minding and Managing the Potential Threats around Us; Ripp, S., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2011.

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decomposing waste gas molecules. For example, metal oxide nano-catalysts (e.g. DeNOx catalysts) could be applied for the removal of nitrogen oxides from fossil fuel power plant emission gases (*11*). In green manufacturing, nanotechnology can increase resource-use efficiency and minimize generation of polluting waste products, thus mitigating greenhouse gas emissions. For example, nanomaterials can help create harder alloys and ceramics for cutting tools to increase the efficiency of manufacturing. Applying nanomaterials to the manufacture of wind turbine blades can make them stronger, lighter, and more durable. As a result, the blades can easily accelerate to top speed even in light winds to produce more electricity. The use of lightweight materials could lower the amount of fuel needed for transportation while improving the safety of ground and air transportation. In the field of green energy, the use of nanocatalysts can produce cleaner, less costly, and environmentally friendly petroleum refining. Nanotechnology can be used to create more efficient and productive batteries and fuel cells. For example, nanostructured electrode materials can improve the performance of lithium ion batteries, and nanoporous silicon and titanium dioxide can be used in advanced photovoltaic cells and hydrogen production (*77*). Fuels themselves can burn cleaner due to better filtration enhanced by nanotechnology or addition of nanocatalyst (e.g., CeO_2) (*78*, *79*).

4. Environmental Risks

More than 1,300 nano-products (including sunscreens, cosmetics, fabrics, fertilizers, food products, industrial catalysts, surface coatings, paints, and building materials) are currently on the global market, and this figure will reach 3,400 by 2020 (*80*). Nanomaterials could enter the ecological environment as a result of these products' manufacture, use, and/or disposal. The concerns about the potential toxicity of nanomaterials are based on not only their chemical composition but also their unique surface, catalytic, and magnetic properties and how these properties are expressed in biological systems and in the environment to exert adverse effects (*81*). The interactions between nanomaterials and the ecosystem (including food crops) are poorly understood. It is thus difficult to predict the fate and consequences of the nanomaterials introduced into the environment (*82*).

4.1. Soil Ecosystem

Nanoparticles can enter soils when they are used for subsurface remediation (e.g., nano-iron) or when their wastes are disposed or recycled (e.g., using nanosilver-containing biosolid as fertilizer). As a result, nanomaterial-based practices might cause their own set of serious ecological threats that are difficult to mitigate (*10*, *17*, *83*). Many types of nanoparticles (e.g., silver, titanium dioxide, fullerenes, and carbon nanotubes) have been found to have antimicrobial properties associated with exposure. These antimicrobial properties have led to concerns that they may shift into microbial populations and disrupt signaling between nitrogen-fixing bacteria and their plant hosts. Any significant disruption

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of nitrogen fixing could retard plant growth and have serious negative impacts for the functioning of entire ecosystems. For example, high levels of exposure to nano-alumina (13 nm in diameter) have been found to slow root growth of five commercial crop species in a soil-free exposure medium, while larger alumina particles (200-300 nm) had no such effect (*84*). The same research also showed that organic coating of the alumina nanoparticles with phenanthrene lessened the inhibition effect on root growth. Cañas et al. (*85*) reported that non-functionalized SWCNTs inhibited the root length of six crops (cabbage, carrot, cucumber, lettuce, onion, and tomato) more than the SWCNTs functionalized with poly-3-aminobenzenesulfonic acid (PABS) at a ratio of PABS to CNTs of 65:35 (w/w). Lee et al. (*86*) found that copper nanoparticles were bioavailable and toxic to *Phaseolus radiatus* (mung bean) and *Triticum aestivum* (wheat). However, a cupric ion released from copper nanoparticles had negligible effects, indicating that the apparent toxicity clearly resulted from copper nanoparticles. Similar results were reported by Shah and Belozerova (*87*) regarding the effects of silica, palladium, and gold nanoparticles on soil microbial communities and the germination of lettuce seeds. While we are aware of these negative effects, using knowledge of nanoscale signaling and nanotechnology to mange ecosystems might mitigate deleterious effects. For instance, nanotechnology could be used to regulate the vital rates of individuals within the ecosystem, which could control population growth by harnessing key environmental signals that govern the rate-limiting process. However, to achieve this goal, both short-term and long-term research is needed to identify the adverse and beneficial perturbation of nanomaterials at all scales of organization (individual, population, community, and ecosystem).

4.2. Food Web

Nanoparticles are easily aggregated and/or absorbed on environmental media resulting in reduction of mobility, but they still have the potential to be taken up by sediment-dwelling organisms (*12*). Nanomaterials usually affect the food web through cytotoxicity and bioaccumulation. Ecological studies demonstrate that some nanomaterials are toxic to aquatic organisms at low levels on the food web (*88–92*), particularly materials containing silver. Nanosilver-containing products (e.g., soap, clothing, and computer boards) can leak silver out into the wash water, threatening aquatic life, such as prokaryotes, invertebrates, and fish. For example, nanosilver can enter cells to cause severe developmental problems in zebrafish embryos (*93*). Aqueous suspensions of nanoscale TiO_2 (*94–96*), ZnO (*94*, *97*), and CeO_2 (*98*) also show the potential for cellular damage in both prokaryotic and eukaryotic cell cultures, apparently through the generation of reactive oxygen species (ROS) (*94*).

Accumulation of nanoparticles or their dissolved elements in food web organisms is another concern but the associated potential toxicity has not been well understood. A number of studies indicate that nanoparticles may not be easily transferred in biological systems. Holbrook et al. (*99*) reported that carboxylated and biotinylated quantum dots can be transferred to higher trophic organisms (e.g., rotifers) through dietary uptake of ciliated protozoans. However, the particulate nature of nanomaterials limits their distribution in the food chain. Should these materials make their way into the environment in significant amounts, they may bioconcentrate to some degree. However, it is anticipated that they would not bioaccumulate or biomagnify in the food chain because they are still solid particles and may not become truly dissolved (which is prerequisite for conventional toxics). The study by Holbrook et al. (*99*) shows that there was limited accumulation (bioconcentration) of quantum dots in the ciliates and enrichment (biomagnification) of quantum dots was not observed in the rotifers. In addition, bioaccessibility of nanoparticles to organisms is limited because of the tendency for nanomaterials to aggregate and sorb onto environmental media. Therefore, nanomaterials might not pose a significant risk to the environmental organisms as a result of a passive cumulative mechanism (*100*). Nevertheless, an exception may occur if a nanomaterial contains elements or compounds that are already known to be either extremely toxic or biomagnify, such as mercury, cadmium, and selenium.

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4.3. Aquatic Ecosystem

Release of nanomaterials into the water environment may pose a significant risk to aquatic organisms such as fish and wildlife (*101*). The catalytic properties of nanoparticles that degrade pollutants can also induce a toxic response when taken up by the cells of organisms (*91–95*, *102*). A host of factors determines exposure and toxicity of nanomaterials to aquatic receptors: the type of receptor, its habitat, the duration of exposure, age, gender, sensitivity or tolerance, adaptive mechanisms, and the properties of the nanomaterials in question. For aquatic receptors, water will be the obvious route of exposure and the respiratory system (namely the gills) is expected to be the target. Many studies have demonstrated the aquatic hazards of nanomaterials. For example, fullerenes (C60), a spherical particle with a diameter of ~1 nm, has little impact on the structure and function of soil microbial communities and associated processes (*103*). However, C60 in water suspensions exhibits relatively strong antibacterial activity (*104*) and it has been found to kill water fleas and cause brain damage in largemouth bass, which is a species accepted by United States regulatory agencies as a model for defining eco-toxicological effects. SWCNTs display greater pulmonary toxicity than carbon black nanoparticles (*105*, *106*). Impurities (Co and Ni) contained in SWCNTs was found to be toxic to zebrafish embryos (*107*). Mouchet et al. (*108*) found that multiwalled carbon nanotubes (MWCNTs) inhibited growth of larvae at 50 mg/L in water but genotoxicity was not noticed. Shvedova et al. (*109*) reported unusual inflammatory responses to specific nanomaterials in mammals, suggesting that some nanomaterials may injure organs by novel mechanisms. Quantum dots (e.g., CdTe) are also toxic due to their release of toxic heavy metals to the ecosystem, their small size, and their reactive surface chemistry (*110*, *111*). Quantum dots could generate ROS by transferring energy to nearby oxygen molecules, leading to cell inflammation, damage, and death (*112*). Choi et al. (*19*) reported that quantum dots could induce cell death by lipid peroxidation of human neuroblastoma cells. Gagné et al. (*20*) found that CdTe quantum dots are immunotoxic to freshwater mussels and can cause oxidative stress in gills and DNA damage. Therefore, nanoparticles could poison aquatic life, harm human health, and contribute to the rise of antibiotic resistance. Currently, there is no effective way of testing for nano-waste in the water and cleaning up such pollution. Therefore, caution is recommended in the use and disposal of manufactured nanomaterials to prevent unintended environmental impacts.

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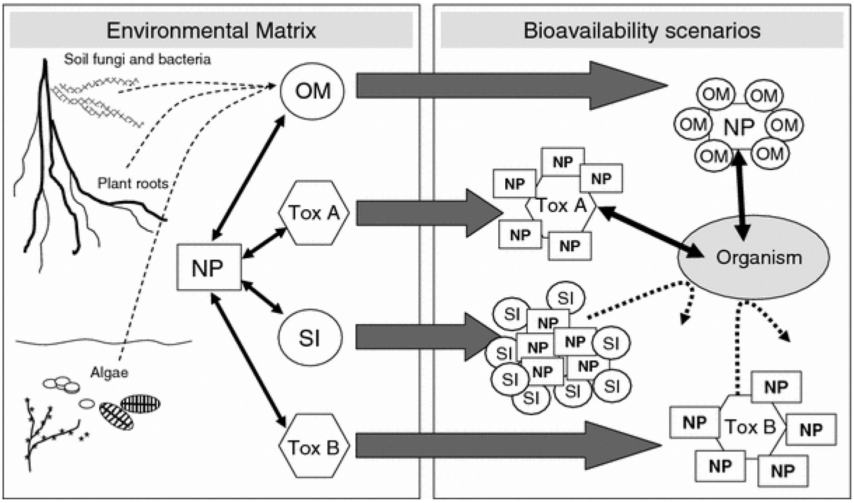

Figure 4. Scenario of nanoparticles' interactions with toxicants (Tox A and B), salt ions (SI), and organic matter (OM) such as humic acids or compounds released by plants, fungi, bacteria, and algae. Some compounds present in environmental matrices might increase the nanoparticle's stability (i.e., OM) and thus bioavailability (represented as solid arrows entering organisms), whereas others (salt ions) might foster the aggregation of nanoparticles, thus reducing their bioavailability (represented as dotted arrows not entering organisms), or physically restraining nanoparticle-organism interactions. In other cases, nanoparticle bioavailability might be either increased or decreased. (Reproduced with permission from Navarro et al. (28). Copyright 2008 Springer Inc.)

4.4. Air Pollution

Applications of nanotechnology may cause new problems with air quality. For example, a few types of metal-based nanoparticles, including cerium oxide (*78*, *79*), platinum (*113*, *114*), palladium (*115*), and aluminum (*116*), are being used as diesel fuel additives to improve combustion efficiency and decrease emissions. A study employing a cerium additive has shown that cerium can significantly change the physical and chemical properties of diesel exhaust emissions, resulting in increased levels of toxic air chemicals such as benzene, 1,3-butadiene, and acetaldehyde (*117*). It was reported that platinum nanoparticles (0.8-10 nm) are released from car catalysts during their life-time (*113*). Presence of these nanoparticles in the air may be harmful to the health of humans and animals. CeO_2 nanoparticles were found to produce significant oxidative stress in the lung cells after penetrating into the cytoplasm, resulting in dose-dependent cell membrane damage and cell death (*118*, *119*). Nanodispersed platinum elements can be transferred to animal tissues (*120*). Therefore, there is increasing concern about the environmental hazards of increased use of nanomaterials as catalysts in vehicle fuels (*121*). It has been predicted that ambient air levels of cerium would increase with increasing use of cerium additive in fuels (*112*). However, detailed toxic and health impacts of these changes in diesel exhaust currently remain unknown. Integrated exposure and toxicological studies are needed in the context of the application environment.

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4.5. Interaction with Aqueous Pollutants

Concerns about the aquatic toxicity of nanomaterials can be put in a broader perspective. The high sorption capacity of nanoparticles that are used to remove organic and inorganic pollutants from groundwater may sequester and transport other pollutants in the environment (*12*, *24*, *122*), causing colloid-facilitated contamination. On the other hand, the pollutants released to water by industry may interact with nanoparticles to change their toxicity and uptake by organisms (Figure 4). This phenomenon may occur in many environments, such as at water treatment plants, in paint production, and in natural water systems where chemically complex mixtures are present. Studies by Baun et al. (*123*) indicate that the presence of C60 nanoparticles increase the toxicity of phenanthrene to algae *P. subcapitata* and the zooplankton *D. magna* at lower concentrations, but decrease the toxicity in freshwater crustaceans, in spite of the 85% sorption of phenanthrene to C60. However, C60 made pentachlorophenol less toxic to both algae and crustaceans and it showed little effect on the toxicity of atrazine and methyl parathion (*123*). Nanoparticles also affect the rate and quantity of the pollutant taken in by the organisms. For example, nanoscale TiO_2 can increase the accumulation of arsenic (*124*) and cadmium (*125*) in carp and the toxicity of arsenic (V) (*126*). Nanoscale alumina was found to increase the bioavailability of phenanthrene to plant roots (*82*). Nanoparticles, particularly those used to degrade pollutants or kill pathogens (*127*), may induce other chemical transformations (*122*). For example, nano CeO_2 (a strong nano-oxidant) could decarboxylate and polymerize some organic molecules (*128*). Its release into the wastewater treatment systems may thus affect the carbon chemistry associated with the fate of many organic pollutants and microbial activities (*122*, *129*). Overall, little is currently known about the toxic effects associated with the interactions between manufactured nanoparticles and aqueous pollutants. Therefore, assessment of the environmental risks of nanotechnology must consider specific environmental conditions. In addition, aquatic substances may affect the fate and transport of nanoparticles, thus changing their exposure and toxicity to organisms (*130*). For example, aluminum sulfate, which is used in waste water treatment for efficient removal of particles, pollutants, and pathogens, may cause coagulation of nanoparticles (*131–133*). Once they form large agglomerates, nanoparticles would eventually settle out of the water body into soil or sediment. Over time, these agglomerations might bind irreversibly to environmental matrices. A study by

Cheng et al. (*105*) shows that aggregates of SWCNTs added to zebrafish embryos reduced hatching rates. On the other hand, natural environmental molecules (e.g., dissolved organic matter) may reduce aggregation of nanoparticles by increasing electrostatic repulsion, thus increasing their exposure and toxicity to organisms (*130*, *134*).

5. Uncertainty in Risk Assessment of Nanoparticles

The increased use and disposal of products containing manufactured nanomaterials will inevitably result in their accumulation in soil, water, air, and organisms via direct inputs and/or runoff from contaminated sites (*15*). Assessment of their exposure and hazards in the real environment through the life cycle of nano-products must rely on standardized testing protocols (including monitoring tools) and integrated risk analysis methods (*100*). LCA is a well-established methodology for evaluating the environmental impact of products, materials, and processes in terms of specified impact categories (*135*, *136*). However, uncertainty exists when traditional LCA is applied to assess nanoparticle risks (*137*). In general, uncertainties are involved with system boundary selection, inventory data collection, and toxicity assessment. At present, there is a large data gap in LCA of nanoparticles, such as material properties, mobility, bioavailability, bioaccumulation, and potential toxicity. The majority of companies do not perform any form of risk assessment (*138*), and few studies incorporate uncertainty through a complete LCA (*139*). To improve the LCA-based risk assessment, we suggest:

- Developing the ability to monitor and characterize the behaviors and toxicity of nanomaterials in the complex environment. To this end, advanced analytical techniques (e.g., functional genomics, proteomics, and metabolomics) should be used within a number of model systems (e.g., mammalian cell lines, zebrafish, daphnia, and representative plant and microbial models) (*140*) in addition to developing validated models for predicting the release, transport, transformation, accumulation, and uptake of manufactured nanomaterials in ecosystems. This approach allows identification of potential genotoxic effects (*141*) and has the potential to provide a global assessment of the biological response to a novel nano-toxicant. It is thus very useful for determining the potential toxicity of nano-products in varying lengths of time. In the real environment, breakdown and/or biological deactivation of nanomaterials, such as coated or encapsulated nanoparticles, is also a factor that affects nano risk assessment. The LCA of nanoparticles should first be performed on the behavior and transformation of nanoparticles in the most susceptible communities of the ecosystem.
- Creating a nanoparticle database with information on the properties of different manufactured nanomaterials. Such a database should assist in categorizing nanoparticles with respect to, for instance, chemical properties, toxicity, and consumer use. The database could have an

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international scope because international trade of nano-products affects where the nanowastes are eventually disposed. Statistical information should also be included to propagate uncertainty and variability involved in various LCA stages.

- Establishing an early warning system against the adverse effects of nanoparticles from commercial products and generated wastes, such as barcode labeling of nanomaterial-containing products, recommendation of disposal methods, and reporting of nanowaste release to the public.
- Providing risk management strategies and/or guidelines to help industry address public concerns on potential risks of nanoparticles and nanowastes treatment. Examples include collection and storage of toxic nanoparticle-containing wastes in a way that lowers their hazards and/or the exposure potentials. The strategies or guidelines should facilitate a balance between regulation to protect human and environmental safety and support for nanotechnology applications.

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6. Closing Remarks

While nanotechnology offers cleaner, cheaper, faster, and smarter approaches, it may introduce a whole new range of ecological risks by excessive exploitation of natural resources (e.g., metals) and releases of a large amount of non-biodegradable nanowastes to the environment. The risks might not occur immediately but may result in serious problems in the long term. The public and policymakers thus have good reasons to suspect the industry claim that nanotechnology will solve our environmental problems (e.g., pollution, water scarcity, and greenhouse gas emissions). From a historical perspective, many past technologies (e.g., automotive) that offered efficiency gains failed to translate into net environmental savings because of a lack of multi-dimensional assessment of life-cycle impact. In fact, driven by short-term profits, nanotechnology has rushed ahead of our understanding of the potential risks and the establishment of environmental safety regulations. Although currently limited data indicate that manufactured nanoparticles might not cause high hazards to the ecosystem and human health, rapid development and expanding use and disposal of next generation nano-products very likely pose complex risks. Therefore, it is necessary to take precautionary approaches to ensure that benefits outweigh the risks of each new nanotechnology before it is used on a large scale. Meanwhile, a social or commercial system alerting potential risks should be established based on the life-cycle analysis of potential hazards of nanomaterial-containing products. Toxic nano-wastes should be collected and disposed in a way different from conventional waste treatments.

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Chapter 4

Environmental Fate, Transport, and Transformation of Carbon Nanoparticles

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Mass production of carbon nanoparticles (CNPs) is rapidly growing, and their entry to the environment is inevitable. Such releases of CNPs may cause undesired/unforeseen risks to the environment and human/wildlife health. A scientific assessment of such risks requires a thorough understanding of the environmental behaviors of CNPs, such as their fate, transport and transformation. This chapter presents a review on the important processes that govern the environmental behaviors of CNPs in natural aquatic systems, including aggregation, sorption, transport in porous media, and biotic and abiotic transformations.

1. Introduction

Carbon nanoparticles (CNPs) are a family of nano-sized molecules composed almost entirely of carbon, with fullerene C_{60} and carbon nanotubes (CNTs) being the two most common types. C_{60} is a hollow spherical molecule, about 1 nm in diameter, comprised of 60 sp2 carbon atoms. CNTs are made in two principal classes: single- and multi-walled. Single-walled carbon nanotubes (SWCNTs) are one-layered graphitic cylinders with 1 - 5 nm diameters (*4*); whereas multi-walled carbon nanotubes (MWCNTs) comprise 2-30 concentric graphitic cylinders with outer diameters commonly between 10-100 nm. The lengths of CNTs vary widely, ranging between 10 nm to more than 1 cm (*4*). Several techniques, e.g., arc discharge, laser ablation, and chemical vapor deposition (CVD), have been used in CNP synthesis (*10*). Theoretically, pristine CNPs are purely composed of carbon atoms; however, in practice, defects cannot be avoided during synthesis

In Biotechnology and Nanotechnology Risk Assessment: Minding and Managing the Potential Threats around Us; Ripp, S., et al.; ACS Symposium Series; American Chemical Society: Washington, DC, 2011.

and subsequent purification processes. These defects usually impart the CNPs with hydroxyl or carboxyl surface functional groups. In addition, two types of methods are often used to modify CNP surfaces for increased solubility and biocompatibility, including: 1) strong oxidative treatments to create hydrophilic surface functional groups, and 2) the use of amphiphilic polymers or surfactants to wrap around and solubilize CNPs.

Given the probable widespread application of manufactured CNPs, large-scale environmental release is possible. The total production capacity for nanocarbon products, including SWCNTs, MWCNTs, fullerenes, graphene, carbon nanofiber and nanodiamonds increased from 996 metric tons in 2008 to more than 2190 tons in 2009, and 4065 tons in 2010. The production capacity is expected to exceed 12,300 tons in 2015, reaching a compound annual growth rate of 24.8%. The growth is chiefly driven by multi-walled carbon nanotubes. World production capacity for multi-wall carbon nanotubes exceeded 390 tons in 2008, 1,500 tons in 2009, and was expected to exceed 3,400 tons in 2010 (*11*). CNPs may enter the environment through incidental release during manufacturing, transport and product use, or through waste disposal and decomposition (*12*, *13*). Understanding the environmental behavior of CNPs is of paramount importance for an accurate environmental risk assessment. This review focuses on the processes that govern the important environmental behaviors of CNPs in natural aquatic systems. Aggregation, a process that controls the distribution of CNPs between solid and aqueous phases, is first discussed below. Sorption of CNPs on solid phases, a process that influences both CNP phase distribution and transport, is followed. The studies on transport behaviors of CNPs in porous media are then reviewed. Finally, recent investigation on transformation of CNPs under biotic and abiotic conditions is summarized.

2. Aggregation

Fullerene C_{60} has limited solubility in some organic solvents (*14*) and is almost insoluble in water (*5*). Similarly, pristine CNTs could sparsely disperse in some organic solvents but not in polar solvents (*15*). As nanoscale particles (i.e., at least one dimesion is below 100 nm), CNPs can undergo Brownian motion and thus remain suspended in water over certain time scales as long as the settling velocity is equal to, or less than, the Brownian displacement. On the other hand, CNPs have large specific surface areas which are hydrophobic in nature, and are thus easy to aggregate leading to their settling from water. Aggregation is therefore a crucial process governing the phase distribution of CNPs. We will first in the following subsection (2.1) discuss the fundamentals of colloid science in relation to CNP aggregation, and then in subsequent subsections review observations on the effects of various system conditions on CNP aggregation.

2.1. Colloidal Nature

Nanoparticles are essentially colloids, although towards the lower end of the colloid size range (Figure 1). As such, CNPs dispersed in water form a colloidal

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dispersion which differs from a true solution in nature. A colloidal dispersion refers to one phase (e.g., solid) homogeneously distributed in another phase (e.g., water) (*6*).

Colloidal dispersions are dynamic non-equilibrium systems, and are often sensitive to physical or chemical disturbances, which result in the aggregation of particles (*16*). There are two major steps involved in aggregation: particle transport (collision) and attachment (*6*, *17*). The first step can be originated from three fundamental processes: Brownian diffusion of particles leads to perikinetic aggregation, shear flow transport of particles at different velocities causes orthokinetic (shear) aggregation, and particles of different size or density undergo differential settling (Figure 2). After initial aggregation, particle-cluster and cluster–cluster aggregation processes also take place (*6*).

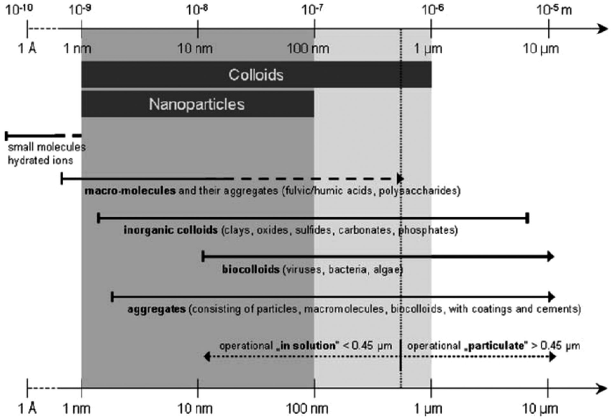
10-10
10-9
10-8
10-7
10-6
10-5 m
1 Å
1 nm
10 nm
100 nm
1 µm
10 µm
Colloids
Nanoparticles
small molecules
hydrated ions
macro-molecules and their aggregates (fulvic/humic acids, polysaccharides)
inorganic colloids (clays, oxides, sulfides, carbonates, phosphates)
biocolloids (viruses, bacteria, algae)
aggregates (consisting of particles, macromolecules, biocolloids, with coatings and cements)
operational „in solution" < 0.45 µm
operational „particulate" > 0.45 µm
1 Å
1 nm
10 nm
100 nm
1 µm
10 µm

Figure 1. Size domains and typical representatives of natural colloids and nanoparticles. The vertical line represents the operationally defined cut-off given by filtration at 0.45 µm. Used with permission (3).

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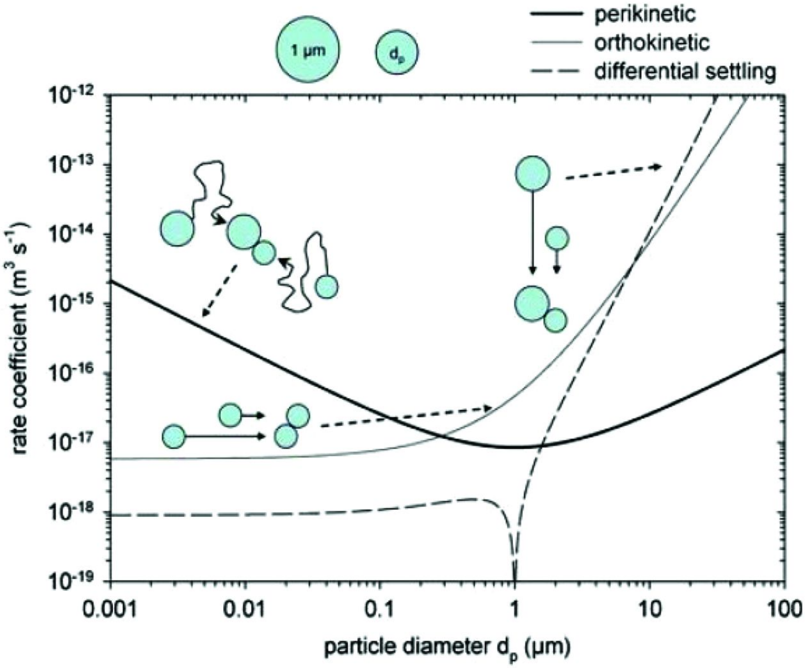

Figure 2. The three collision mechanisms and associated rate coefficients for the aggregation of 1 μm particles with particles of diameter d_p; the temperature is 12°C, the particle density 2.6 g/ml, and the shear rate 35/s. The cartoons represent the processes of perikinetic, orthokinetic, or differential settling, respectively. Dotted arrow indicates the graph relating to each cartoon (process). Used with permission (6). (see color insert)

Generally the Brownian motion applies to a particle range of 1 nm—1 μm. When the particle size is beyond this range, the particles (or aggregates) begin settling. The settling rate for a spherical or near-spherical particle is proportional to the square of the particle diameter. When applied to non-spherical particles like CNTs, equivalent diameter can be calculated in terms of equivalent settling velocity or diffusivity. It is suggested that CNTs shorter than 500 nm can be simulated by ellipsoids (*18*), and the radius for a sphere of equivalent diffusivity can be calculated by Equation 1.

$$r_{eq} = a\,\frac{16}{3}\,\frac{s^2}{2s + (3s^2 - 1)Z} \qquad (1)$$

where $s = \sqrt{1 - \left(\frac{a}{b}\right)^2}$, *a* and *b* are the radii of major and minor axes of the ellipsoid, respectively. *Z* is a function of *s* (*18*). Nonetheless, the effect of CNT shape on the aggregation and settling is still largely unknown and under debate.

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Pristine CNPs have a strong tendency to attach and aggregate when they collide with each other as a result of attractive van der Waals forces. Experiments indicated that raw MWCNTs settled more rapidly than carbon black and activated carbon particles. This may be attributable to the much greater aspect ratio of MWCNTs (1:1,000), allowing for multiple contact points between particles, greater entanglement, and increased van der Waals forces, leading to aggregates of increased mass (*19*). Introduction of negative surface functional groups during CNP purification or modification tends to mitigate aggregation. Different types of surface functional groups can be added to CNPs during various purification and dispersion processes (*20*). These purification processes and their outcome will be discussed in greater detail later in the transformation section in this chapter. Such functional groups are in general hydrophilic and acidic (e.g., carboxyl, hydroxyl and carbonyl groups) (*7*, *8*, *15*, *21*). Carboxyl groups, in particular, have low pKa values (~3.5), and are thus dissociated around common aqueous conditions rendering negative charges on CNP surfaces, although the charge densities may vary depending on the CNP synthesis and purification procedures. Smith et al. (*12*) found that the zeta-potential is proportional to the amount of carboxyl groups on CNT surfaces ($R^2 = 0.89$). It was also found that up to six electrons can be accommodated in the lowest unoccupied molecular orbital (LUMO) of C_{60} despite its resistance to oxidation, which opens the route to covalent surface addition (*22*). Even in pure water, extended aqueous exposure can cause the initially hydrophobic C_{60} to form water-stable aggregates with externally positioned polar functional groups (*23*). These amphiphilic fullerene derivatives contain polar functions, such as hydroxyl, carboxyl and amino groups (*24*). Regardless of preparation methods, the surface charge of fullerenes and their aggregates are usually negative as indicated by their negative electrophoretic mobility or zeta potential, although the mechanisms are not fully understood yet (*25*). One explanation is that the core hydrophobic C_{60} molecules are likely cloaked by a polar shell formed via localized surface hydrolysis (e.g., $C_{60} + H_2O \leftrightarrow C_{60}(OH)^- + H^+$) (*26*).

It has been found in several studies that the attachment efficiencies of both CNTs and fullerenes can be fairly well modeled using the Derjaguin—Landau—Verwey—Overbeek (DLVO) theory that describes the interactions of charged spherical colloidal particles.

2.2. Effect of Cation Concentration and Valence

It is believed that the charges on CNP surfaces lead to repulsion forces that prevent CNP aggregation, and the surface potential of CNPs is correlated to their stability in aqueous systems (*5*, *12*, *25*, *27*). It thus follows that any factor that can influence CNP surface potential may result in changes in the stability of CNPs in water.

Consistent results have been reached in different studies that cations present in solution facilitated CNP aggregation (*5*, *9*, *25*, *27*, *28*). The cause has been rationalized, based on DLVO theory, to be the compression of the electric double layers (EDL) around CNPs by solution cations. Although DLVO theory was derived for ideal spherical particles with evenly distributed surface charge and other surface properties, the aggregation behavior of CNTs can still be reasonably well modeled by DLVO theory in many aspects despite of their cylindrical shapes (*27*). In contrast to CNTs, fullerene and fullerene aggregates are more sphere-like, which renders them more amenable to measurement techniques and modeling based on electrophoretic mobility (*5*, *27*) or zeta-potential (*25*, *27*). As the concentration of electrolytes increased, the electrophoretic mobility (*5*, *27*, *29*) or zeta-potential (*27*, *30*) of CNPs became less negative. For example, the zeta-potential of fullerene nanoparticles decreased linearly with the concentration of KCl (*30*). This suggests that the repulsive force between CNPs is reduced, and the attachment efficiency between CNPs would increase due to the decreasing energy barrier to aggregation. Once the concentration of electrolytes reaches or exceeds a certain value, i.e., the critical coagulation concentration (CCC), the particle surface charge becomes completely screened, thus eliminating the energy barrier to aggregation so that attractive forces between particles, e.g., van der Waals force, become dominant.

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The electrolytes commonly used in most studies cited above are NaCl, KCl, $MgCl_2$ and $CaCl_2$. Aggregation kinetics of the CNPs exhibited slow (reaction-limited or unfavorable) and fast (diffusion-limited or favorable) regimes in the presence of these cations, the intersection of which is the CCC (Figure 3) (*5*, *27*, *28*). The ability of divalent cations, e.g., Mg^{2+} or Ca^{2+}, to induce aggregation is dramatically stronger than that of monovalent cations, e.g., Na^+ or K^{+-} (*5*, *9*, *27*). The CCC of Na^+ for fullerene aggregation was 120 mM (*5*), while that of Ca^{2+} was 4.8 mM (*5*). According to the Schulze-Hardy rule, CCC is proportional to Z^{-6} for surfaces with high charge densities, or Z^{-2} for those with low charge densities (where Z is the counterion valence) (*5*, *9*, *31*). Observations with colloidal particles showed the CCC value dependence on Z ranged between Z^{-6} and Z^{-2} (Figure 4) (*17*). In a study with acid-treated MWCNTs, Smith et al. (*27*) found that the ratios of CCCs of $MgCl_2$ and $CaCl_2$ over that of NaCl were $2^{-5.7}$ and $2^{-6.3}$, respectively, very close to the theoretical value of 2^{-6} or 1/64. However, this theoretical ratio of 1/64 is not only determined by the valence of cations, but also the symmetry of electrolytes and the shape of CNPs. Researchers showed that for symmetric 2:2 electrolytes such as $CaSO_4$ the ratio was 1/64, whereas for asymmetric 2:1 electrolytes, such as $CaCl_2$, the ratio should be 1/42 (*31*, *32*). Chen et al. (*31*) found the ratio of CCC values for colloidal particles with $CaCl_2$ over NaCl was 1/40, consistent with the 1/42 prediction. Moreover, there were some experimental results not in good agreement with the 1/64 value, for example, 1/25 or 1/10 for $CaCl_2$ (*5*) and 1/17 for $MgCl_2$ (*27*). These discrepancies may be caused by different surface charge densities.

While mono- and di-valent cations have large difference in CCCs, the difference between cations with the same valence is minor. The CCC values for an acid-treated MWCNTs (with carboxyl groups on surface) were 1.8 mM for $MgCl_2$ and 1.2 mM for $CaCl_2$ (*27*). Moreover, the type and valence of anions had little influence. The CCC values for the acid-treated MWCNTs were 93 mM Na^+ in the form of NaCl and 98 mM Na^+ for Na_2SO_4 (*27*).

Although solution ionic conditions strongly influence the electric interactions between CNPs, the van der Waals forces are quite independent of solution conditions and CNP surface chemistry. Hamaker constant, a parameter delineating van der Waals potential between two particles, does not change with solution conditions. The fitted Hamaker constants from two experiments investigating the aggregation of fullerene nanoparticles in aqueous medium were 6.7×10^{-21} J at pH 5.2 ± 0.1 (NaCl concentration from about 50 mM to 500 mM) (*5*) and 8.5×10^{-21} J at pH 5.5 (KCl concentration from about 20 mM to 1000 mM) (*25*).

One additional point to be mentioned is that CNTs can be individually dispersed in water, at least for a large portion, while the water suspension of fullerenes is indeed their aggregations (nC_{60}) instead of individual C_{60}. The clusters have properties different from individual or bulk C_{60}, which limits the application of individual C_{60} in aquatic systems.

Comparing the CCC values of fullerenes, SWCNTs, and MWCNTs provides information on the relative aqeous stability of these CNPs. The CCC values were 20 or 37 mM Na^+ for SWCNTs, 93 or 98 mM Na^+ for MWCNTs, and 120 or 160 mM Na^+ for fullerene (*5*, *24*, *27*, *33*), indicating an increase of stability in this order. The CCC values of the divalent cation Ca^{2+} for SWCNT (~2 mM) (*32*) and MWCNT (1.2 mM) (*27*) were similar with each other, while that for fullerene was higher (6.1 mM) (*24*, *33*).

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2.3. Effect of pH

CNP aggregation is also strongly influenced by pH, mainly because of the protonation/deprotonation of surface functional groups. Dissociation of surface functional groups contributes to surface charges. For example, the zeta-potential of fullerene nanoparticles became more negative when pH increased from 2 to 12, indicating more charges added (*25*), which led to smaller aggregate sizes and more stable suspension (*28*). Similar observations were made with CNTs (*15*). An increase in solution pH from 3 to 11 resulted in a substantial (over 2 orders of magnitude) decrease in MWCNT aggregation kinetics (*27*). Acid-treated MWCNTs were unstable at pH 0 but the stability increased when pH increased from 4 to 10 (*27*, *34*). However, the electrophoretic mobility did not change much when pH was above 6 (*27*), indicating that electrophoretic mobility is not necessarily consistent with colloidal stability (*12*, *27*).

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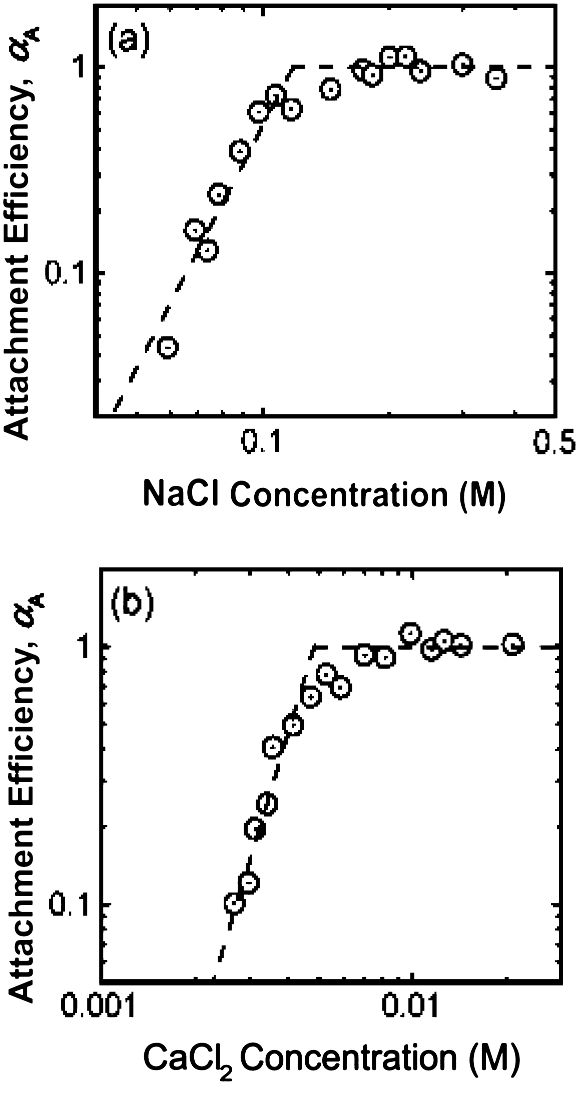

Figure 3. (a) Attachment efficiencies of fullerene nanoparticles as a function of NaCl concentration at pH 5.2. The critical coagulation concentration (CCC) based on these data is 120 mM NaCl. (b) Attachment efficiencies of fullerene nanoparticles as a function of $CaCl_2$ concentration at pH 5.2. The CCC based on these data is 4.8 mM $CaCl_2$. The lines (used as eye guides) are extrapolated from the reaction-limited and diffusion limited regimes, and their intersections yield the respective CCC. Used with permission (5).

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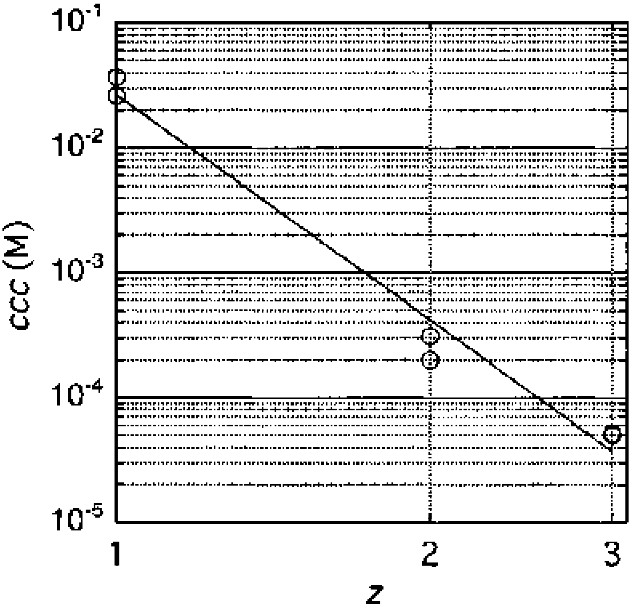

Figure 4. Double logarithmic plot of the critical coagulation concentrations (CCC) against the cation valance. The solid line has a slope of -6. Used with permission (9).

2.4. Effect of CNP Preparation Methods

Different CNP synthesis approaches and the methods used to prepare CNP suspension will result in different CNP surface chemistries, and consequently different aggregation behavior. Several methods have been used to prepare fullerene water suspensions. One is the solvent exchange method comprising two steps: 1) dissolve fullerene in an organic solvent, e.g., tetrahydrofuran (THF) (*20*, *25*, *35*) or toluene (*5*, *20*, *25*, *35*), and 2) introduce the mixture into water followed by removing the solvent through distillation (*20*, *35*) or sonication (*5*, *20*, *25*, *35*, *36*). Another method is prolonged stir or ultrasonication of fullerenes in water (*20*, *25*, *25*, *25*, *37*). Dissimilarities were found between the nC_{60} produced by different methods, with respect to size, morphology, charge and hydrophobicity (*20*, *35*, *37*). For example, the relative hydrophobicity expressed as a partitioning coefficient to dodecane from water was 3.6% and 0.8% for nC_{60} prepared by solvent exchange using THF and extended mixing in water, respectively (*20*). Chen et al. (*25*) reported that the CCC value for fullerene stirred in water is 166 mM KCl, which is significantly greater than the CCC value (40 mM KCl) for fullerene suspension prepared by the solvent exchange method with toluene. These results indicate that a fullerene suspension prepared by solvent exchange seemed to be more stable than that prepared by mixing with water. The type of organic solvent used in the solvent exchange also made a difference; e.g., the nC60 clusters prepared in tetrahydrofuran had larger sizes than those prepared in toluene (*35*). Even when the same stir/sonication method was used to prepare fullerene suspensions, the CCC values may still vary with specific procedure parameters (e.g. mixing time, C_{60}-water ratio). Bouchard et al. (*28*) reported a CCC value for fullerene stirred in deionized distilled water for 5 months (100 mg C_{60} in 400 mL water) of 260 mM NaCl, while Chen et al. (*25*) reported that for a suspension prepared by stirring in deionized water for 40 days (1.22 g C_{60} in 1 L water) the CCC was 166 mM KCl. These phenomena can be attributed to the differences in the solvent properties. This led to different solvent-C_{60} interactions, the presence of different residual solvent in the nC_{60} structure, and thus different processes of the nC_{60} cluster formation (*20*, *38*, *39*).

Solvent exchange is not commonly applied in preparing CNTs suspensions, because the solubility of CNTs is very low even in organic solvents. Methods widely used for preparing CNT suspensions include: 1) oxidization (e.g., acid-treatment, ozonation, etc.); 2) sonication; and 3) stirring in surfactant (or NOM) solutions. The first two methods create hydrophilic surface functional groups on CNTs, the effects of which will be discussed in section 5.1.1. CNTs with these hydrophilic groups tend to be more stable than pristine ones, because the more hydrophilic groups, such as hydroxyl and carboxyl groups, increase particle hydration and thus reduce the probability of particle-to-particle attachment during Brownian motion (*19*). Sonication tends to yield less stable CNT suspensions than the acid-treatment method, as indicated by comparison of their CCC values. For example, the CCC value was 20 mM NaCl for a sonicated suspension of SWCNTs, while that was 37 mM NaCl for the suspension of HNO_3-treated SWCNTs (*9*, *33*). The effects of surfactants and NOM on the stability of CNTs in water solutions are discussed in the following section.

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2.5. Effect of Natural Organic Matter (NOM)

Natural organic matter (NOM) ubiquitously exists in natural or engineered aquatic systems. It has been found in many studies that the presence of NOM significantly enhances the stability of CNPs in water (*19*, *20*, *24*, *28*, *35*, *37*, *39*) In the presence of Suwannee River NOM at the concentrations from 1 to 100 mg/L, CNTs and fullerenes were found to be more stable in aqueous phase (*5*, *19*, *24*, *27*, *28*, *33*, *35*, *39*), with aggregation rates reduced and aqueous concentrations increased. Similar results were obtained in aggregation experiments using natural river water containing NOM (*39*, *40*) and soluble soil humic substances (dissolved Aldrich humic acid at 150 mg/L and water-extractable Catlin soil humic substances at 300 mg/L) (*39*). To quantitatively investigate the effect of NOM, an experiment was carried out with various NOM concentrations and a fixed initial MWCNT concentration. It was found that, after 24 hours of settling, the concentration of MWCNTs remaining in water was linearly correlated with the NOM concentration varying from 0 to 100 mg/L (*19*, *39*). In general, the concentration of MWCNT stably suspended in water is dependent on the amount of NOM adsorbed per unit mass of MWCNT (*39*). Similarly, NOM can cause disaggregation of nC_{60} crystals and aggregates under typical solution conditions of natural water (*35*). Moreover, microscopic and dynamic light scattering examination showed the NOM causes disaggregation, resulting in MWCNTs individually dispersed (*39*), and smaller sizes of fullerene aggregates (*35*, *37*, *39*). These effects increased with increasing NOM concentration (*35*).

It is believed that NOM may sorb on CNPs and then exert steric or electrostatic stabilization (*5*, *24*, *28*, *35*, *39*, *39*, *41*). NOM is in nature surface active, and because of this nature, their interactions with CNPs tend to take the form where the hydrophobic moieties of NOM associate with the CNP surface, likely through pi-pi or CH-pi stacking, while their hydrophilic moieties are exposed to the water (*21*, *39–42*). Studies have shown that NOM association on CNTs was an exothermic equilibrium process, similar to spontaneous adsorbate-adsorbent interactions (*39*, *40*), and followed pseudo-first-order rate kinetics (*40*). Hyung et al. (*39*) demonstrated the adsorption of NOM on CNT was proportional to the aromatic carbon content and molecular weight of the NOM. Pi-pi stacking is likely more powerful than CH-pi stacking. The stabilization effect of SDS, which is through CH-pi stacking, was substantially weaker than that of NOM which is through pi-pi stacking (*43*).

The organic matter sorbed on CNPs imposes either steric and/or electrostatic stabilization, depending on their types, i.e., nonionic or ionic (*41*), thus preventing CNPs from attachment and aggregation (*27*, *28*, *33*, *41*). The NOM that is sorbed on CNP surfaces has their hydrophilic portion extending into the solution phase. When two CNP particles approaching, the hydrophilic portion of the NOM interpenetrates and displaces water molecules, leading to steric stabilization (*41*). This stabilization effect is relatively inert to the change of ionic strength in the solution phase. For example, the stability of fullerenes in aqueous phase did not change significantly across a range of NaCl concentrations in the presence of NOM (*24*, *44*). If the organic matter is ionic, both steric and electrostatic repulsion took place and the latter effect was influenced by the ionic strength of the solution (*41*). On the contrary, when adding divalent cations such as $CaCl_2$, bridging effect or complex formation may occur and destabilize CNPs in the presence of NOM (*24*, *41*, *44*).

The aggregation status of CNPs also influences the sorption of organic compounds. For example, theoretical calculations and nitrogen adsorption analysis results demonstrated that aggregation of CNTs led to a significant reduction in surface area (especially for SWCNTs), but a significant increase of pore volume (especially for MWCNTs) due to the interstices trapped in CNT aggregates (*45*). However, the adsorption of organic compounds on CNTs seemed to be controlled to a greater extent by the surface area rather than the pore volume in aqueous systems (*45*).

Solution conditions also affect NOM sorption on CNPs. Higher ionic strength and lower pH both lead to NOM forming more coiled and compact structures (*39*, *46*). This hampers the effect of NOM to stabilize CNTs via steric hindrance. Besides, ions reduce the charge potential of charged moieties on organic molecules. On the other hand, sorption of NOM increases as ionic strength increases or pH decreases, which tend to promote CNT stabilization (*39*, *42*). Thus, the interplay of these two opposite effects determines how ionic strength or pH influences CNP stability in the presence of NOM. Generally, the net result of NOM is to assist CNP suspension. For example, humic acid and alginate (polysaccharide) exerts steric stabilization of CNPs with NaCl and

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$MgCl_2$ present in the solutions (*28*, *33*, *39*). In contrast, in the presence of $CaCl_2$ the aggregation or deposition rates were larger with alginate on SWCNT (only at high Ca^{2+} concentrations) or C_{60} (even at low concentration of 0.3 mM) than the systems without alginate (*28*, *33*, *39*). Such enhanced aggregation was attributed to organic molecule bridging by Ca^{2+} (*33*, *39*). Such bridging effect by Ca^{2+} was also observed in humic acid stabilized C_{60} nanoparticles (*28*). However, such bridging effect was not observed with other di-valent cations, e.g., Mg^{2+} .

2.6. Effect of Surface Functional Groups

The speciation of surface functional groups is another influencing factor. For MWCNTs, Kennedy et al. (*19*) showed that the stabilizing ability of hydroxyl groups is greater than carboxyl groups, whereas Smith et al. (*12*) found that carboxyl groups are more influential than hydroxyl groups or carbonyl groups. It has been reported that the amount of surface oxygen-containing functional groups on CNTs correlated with their surface charge density (*12*), and the increased charge density tends to stabilize CNTs in aqueous phase. Similar to CNTs, the hydrophilic oxygen-containing surface functional groups on fullerenes also help to increase their stability in aqueous phase. For example, the CCC values for PCBM ([6, 6]-phenyl C_{61}-butyric acid methyl ester) modified fullerenes were significantly higher than that of nC_{60} (*28*). When mixing with water, surface hydroxylation of the initially hydrophobic C_{60} molecules appeared to turn the nC_{60} clusters into hydrophilic, which helped stabilizing them in suspensions (*21*).

3. Sorption

Solid phases (sediment or soil) ubiquitously exist in natural aquatic systems. The association of CNPs with these solid phases, or sorption, is another important process governing the partition of CNPs between water and solid phases. The extent to which C_{60} partitions to soil or sediment will influence its bioavailability and toxicity (*39*, *47*), yet sorption of CNPs, especially CNTs, has not been extensively investigated. Sorption of fullerene by soils has been found to follow a linear isotherm with solid phase concentration proportional to aqueous phase concentration (*39*, *48*). Soil organic matter (SOM) plays an important role in the sorption of nC_{60}, and the sorption capacity strongly depends on the organic content of the soil (*39*, *48*). Swelling of clay minerals also contributes to the sorption of C_{60} to soil (*48*). It was found that at low clay to organic carbon ratios, C_{60} sorption was dominated by SOM because much of the clay surface was coated by SOM (*49*). However, at higher clay to SOM ratios ($f_{cm}/f_{oc} > 20$), the sorption of C_{60} by the swelling clay became influential (*48*). This result is consistent with the sorption of organic pollutants to soil. The sorption of C_{60} to SOM was found to depend on the SOM type and properties. If the organic matter is hydrophilic or surface active in nature, steric hindrance may take place and thus exert a stabilizing effect. For example, in a deposition study with silica as a solid phase in solutions containing NaCl, the attachment efficiencies between C_{60} and silica surface was mitigated when the silica surface was pre-coated with

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dissolvable humic acid or alginate (*28*). This result implies that the negatively charged organic moieties or functional groups coated on soil minerals may reduce the sorption of fullerenes by soil.

Studies related to CNT sorption on soil are limited and little is known about the interactions involved in the sorption. One type of soil organic matter (Canadian peat) has been found to sorb acid-treated MWCNTs from solutions containing cations (Na^+), whereas, in the absence of cations, the sorption was not significant (*50*). This was attributed to that the cations caused a decrease in the surface charges of the soil organic material and CNTs, which facilitated interactions between them (*50*). An inorganic clay particle, kaolin, was found to improve MWCNT removal from aqueous phase (*51*), indicating a favorable association between kaolin and MWCNTs. The sorption of functionalized MWCNTs by soils followed a linear sorption isotherm pattern (*52*), whereas modifications of MWCNTs with polyethyleneimine (PEI) procedures to yield positive, negative, or neutral surface charges led to more non-linear sorption isotherm patterns (*52*). Soils also indirectly affect CNT stability in water. In a study investigating the interaction between clay minerals (kaolinite and montmorillonite) and MWCNT suspensions stabilized by surfactant (SDBS, CTAB, and TX100), clay minerals reduced the stability of MWCNTs in two ways: 1) competitive adsorption of surfactants thus reducing their stabilizing effect and 2) bridging between clay mineral and MWCNTs by surfactants (*53*). These effects depend on the properties of surfactants and the sorption capability of clay minerals. Additional research is needed to investigate the sorption effect of different soils or soil components under different solution conditions, and the sorption of SWCNTs.

4. Transport

It is important to understand CNP transport through porous media in order to assess their potential to migrate in natural and engineered systems such as groundwater aquifers and water treatment filters. Most earlier studies of CNP mobility in porous media focused on model solid phases (quartz sand or glass beads) in packed columns (*28*, *54*), with only several exceptions examining heterogeneous soil materials (*55*).

4.1. CNP Transport in Porous Media

Previous studies suggest CNP mobility is governed by physicochemical deposition (filtration) and/or straining (*54*), which are determined by interactions among the CNPs (sorbate), the porous media (sorbent) and the solution (*54*). Heterogeneous solid phases, such as soils and wastewater sludge, comprise both organic and mineral components that have a number of potential sorption sites for CNPs (*56*). Organic matter contains negatively charged carboxyl and phenolic surface functional groups, positively charged sulfhydryl and amino surface functional groups, and regions of hydrophobicity generated by clusters of aromatic and aliphatic moieties (*56*, *57*). While most soil mineral surfaces are hydroxylated and often carry negative charge due to isomorphic substitution, both positive and negative charged sites can exist in metal oxides and along the edges of clays depending on the solution pH. These charged sites of soil as well as surface functional groups on CNPs render the electrostatic interaction one of the major mechanisms governing CNP transport in soil.

Researches using model solid phases, i.e., glass beads or quartz sand, showed that the repulsion between the electrical double layers (EDLs) of CNPs and stationary phases with surface charges of the same sign resulted in stability and mobility. Thus, screening of the EDLs would lead to more deposition and retention of CNPs qualitatively consistent with the conventional colloid deposition theories (*28*, *54*, *55*). When electrolyte (NaCl) concentration increased from 1 mM to 10 mM, the C/C_0 value (the relative effluent concentration) of nC_{60} clusters (168 nm in diameter) decreased from 0.71 to 0.33 (*28*). Similar results have been achieved by several other studies (*28*, *54*, *55*). $CaCl_2$ seemed to be more capable in increasing nC_{60} and CNT retention in columns (*54*, *55*). Under high ionic strength, e.g., ≥ 3.0 mM KCl, deposition (filtration) was the dominant process for CNP retention, while under low ionic strength, physical straining may also play a role in the capture of CNPs (*54*). Incomplete breakthrough of carboxyl functionalized SWCNTs in deionized water was observed ($C/C_0 = 0.90$) with quartz sand packed columns (*54*). On the contrary, in glass beads or Ottawa sand packed columns, minimal nC_{60} retention occurred, and the breakthrough coincided with the nonreactive tracer (Br^-) with deionized water as the mobile phase (*54*). This shows that the straining effect is more likely to take place in CNT transport. Jaisi et al. (*54*) concluded that the shape, particularly the very large aspect ratio of SWCNTs, and their highly aggregated state contribute to the retention of SWCNTs through enhanced straining.

A study using a natural soil as the stationary phase showed that strong physical straining governed and prevented SWCNT transport through the media, which were collectively attributed to the shape and aggregation of SWCNTs, as well as the heterogeneity in soil particle size, porosity and permeability (*55*). This strong retention is insensitive to changes in the ionic strength to above 0.3 mM KCl or 0.1 mM $CaCl_2$ (*55*).

Hydrophobic interaction is another force, in addition to electrostatic, that may influence CNP transport. Fullerene nanoparticles can be functionalized to be more hydrophilic derivatives, i.e., fullerols. It was found that the mobility of fullerols (1.2 nm in diameter, monodispersed) was greater than the nC_{60} cluster (168 nm, monodispersed) (*54*). This may of course result from the difference in particle sizes, because smaller particles tend to be more mobile when all other properties are similar, but differences in hydrophilicity of the two types of particles may also play a role.

It was found in a study with fullerene flowing through a column packed with spherical glass beads at low NaCl concentration (0.001 M) that the breakthrough curve did not monotonically increase with the injection flow (*28*). Instead, the affinity of porous media for nC_{60} increased after approximately one pore volume, followed by increased passage (*28*). This "affinity transition" was attributed to the initial association of nC_{60} to stationary phase created favorable sites for further loading of nC_{60} (*28*). However, the affinity did not continue to increase with more nC_{60} flowthrough but rather decreased, which calls for further investigation into

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these phenomena. When NaCl concentration was high (e.g., 0.01 M or 0.1 M), this affinity transition was greatly reduced or eliminated and the affinity of nC_{60} towards porous media kept decreasing (*28*). Lecoanet et al. (*54*) suggested that this decreasing affinity of SWCNT, fullerol and nC_{60} under 0.01 M NaCl was caused by saturation or blocking of deposition sites within the porous media.

Electrolytes in feed solution influence CNP release from porous media columns. In studies of deposition and transport of fullerene nanoparticles, the retention of CNPs by stationary phases (silica-coated quartz or quartz sands) were partly reversible under high pH of 12 or 10 (*5*, *54*), and when the mobile phase had only a low concentration of monovalent salt (KCl) (*54*). Otherwise, at high monovalent salt concentration or in the presence of divalent salt ($CaCl_2$), the deposition of the fullerenes was mostly irreversible. Introduction of deionized water resulted in a sharply declined breakthrough curve, indicating the fast release of nC_{60} from glass beads and quartz sand (*54*).

The property of the stationary phase is another influential factor with regard to CNP transport in porous media columns. It is generally acknowledged that the stationary phases comprising finer size particles tend to have greater retention ability (*54*). Wang et al. (*54*) found finer Ottawa sand (100-140 mesh) can retain 95% of nC_{60} particles, much more than those by 40- to 50-mesh quartz sands. Under the same flow conditions (1.0 mM $CaCl_2$, Darcy velocity 2.8 m/day), the retention of nC_{60} or MWCNTs in glass bead columns was substantially lower than in the quartz sand columns (*54*). Sectioned column tests showed that the nC_{60} retention by glass beads decreased with distance from the column inlet. In contrast, the retention by quartz sand was relatively constant through the entire column, suggesting that nC_{60} deposition approached a limiting capacity (*54*). Besides, in glass bead columns the nC_{60} retention can be completely recovered by deionized water extraction, whereas the retention by quartz sand was slightly resistant to water extraction (*54*).

The pore water velocity also influences CNP transport in porous media columns, and it is generally inversely related to the retention of CNPs (*54*). Liu et al. (*54*) demonstrated that with greater pore water velocities (>4.0 m/day) MWCNT mobility was greater than that with 0.42 m/day velocity. Fullerenes exhibited similar breakthrough behaviors at a higher flow rate (40 mL/min or Darcy velocity of 0.14 cm/sec), regardless of differences in surface chemistry and sizes of the packing materials (*54*). In addition, the aforementioned affinity transition, in which the affinity between nC_{60} and stationary phase increased at the beginning and switched to decreasing, only occured at high velocity (*28*). However, the removal of fullerene-based nanoparticles was independent of the flow velocity under these conditions (e.g. 10 mM NaCl, pH 7), which suggested that the time scales for fullerene particle attachment or reorganization on the collector surface were greater than the time scale for them to transport to the collectors (*54*). Similar to the results of aggregation studies, humic-like substances largely reduced the retention, while the polysaccharide-based NOM, such as those produced by algae or bacteria, tended to favor deposition of nC_{60} (*54*).

Different transport behaviors were observed with nC_{60} and SWCNTs even at the same column and flow conditions. In a quartz sand column, nC_{60} with diameter

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of 168 nm had lower mobility than SWCNTs in a feed flow containing 10 mM NaCl (*54*); whereas in a soil column, nC_{60} with diameter of 51 nm displayed lower deposition rate and more effective transport than SWCNTs in a flow containing 0.1 to 100 mM KCl (*55*), indicating the importance of the CNP size to the mobility. The nC_{60} transport appeared to be more sensitive than SWCNTs to changes in the cation concentration from 0.03 to 100 mM KCl in the flow solutions (*55*).

4.2. Model Simulation

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Deposition is a crucial process governing the transport of CNPs and can be fitted well to models (*54*). Analogous to aggregation, deposition can be modeled as a sequence of particle transport to the immobile surface or "collector" described by a collector efficiency, η_0, and followed by attachment described by an attachment efficiency, α (*54*). The theoretical single collector efficiency, η_0, is composed of contact efficiencies due to interception (η_I), sedimentation (η_G), and diffusion (η_D). These efficiencies have been well modeled for spherical particles flowing through a system with spherical collectors, which can be applied in simulating spherical or near-spherical particles such as fullerene and fullerene clusters (*58*). However, this may not be readily applicable to CNTs because they are not spherical particles. Small-angle light scattering and ultra small-angle X-ray scattering showed that the morphology of MWCNTs in water were rod-like, and such rod-like morphology was not at the length-scale comparable to individual MWCNTs (from 1 nm to 50 µm), but seemed to be formed by networks of carbon "ropes" enmeshed with polyelectrolyte dispersants (*59*). There has not been any attempt to date to model the transport of such rope-like particles. Liu et al. (*54*) derived a relationship to model the deposition of MWCNTs based on their rod-like morphology to a spherical collector system. The collection efficiency was divided into two parts. The efficiency due to interception (η_I) by "end contact" was defined as:

$$\eta_I = \frac{1}{2}\left(\frac{l}{d_c}\right)^2\left(3 - \frac{l}{l + d_c}\right) \quad (1)$$

The efficiency due to interception (η_I) by "side contact" was defined as:

$$\eta_I = \frac{1}{2}\left(\frac{d_p}{d_c}\right)^2 \left(3 - \frac{d_p}{d_p + d_c}\right) \tag{2}$$

where l is the length of MWCNTs, d_p and d_c are the diameters of the particles and collectors, respectively. The contact efficiency due to sedimentation is calculated by:

$$\eta_G = \frac{\left(\frac{d_p}{l}\right)^{\frac{2}{3}} \left(\frac{0.146\left(\rho_p - \rho\right) g \left(d_p{}^2 l\right)^{\frac{2}{3}}}{\mu v_0 \left[1 - \left(\frac{d_p}{l}\right)^2\right]^{\frac{1}{2}}} \right) \ln\left(1 + \left[1 - \left(\frac{d_p}{l}\right)^2\right]^{\frac{1}{2}}\right)}{\frac{d_p}{l}} \tag{3}$$

The contact efficiency due to diffusion is calculated by:

$$\eta_D = 4.03\left(kT\ln\frac{1 + \left[1 - \left(\frac{d_p}{l}\right)^2\right]^{\frac{1}{2}}}{\frac{d_p}{l}}\right)^{\frac{2}{3}} \times \left(3\pi\mu d_c v_0 \left(\frac{2}{3} d_p{}^2 l\right)^{\frac{1}{3}} \left(\frac{l}{d_p}\right)^{\frac{2}{3}} \left(1 - \left(\frac{d_p}{l}\right)^2\right)^{\frac{1}{2}}\right)^{-\frac{2}{3}} \tag{4}$$

The overall collector efficiency is the sum of the three efficiencies described above:

$$\eta_0 = \eta_I + \eta_G + \eta_D \tag{5}$$

where ρ_p is the MWCNT density, ρ is the fluid density, u is the fluid viscosity, T is the absolute temperature, and k is the Boltzmann constant. To calculate η_G and η_D requires a friction factor; however, this factor has not been developed for a cylindrical particle. Instead, the friction factor developed for a prolate ellipsoid has been employed (*54*). As mentioned above, deposition is the dominant process governing retention of CNTs only at high flow velocity and ionic strength. To compensate, this model was incorporated with a site-blocking term, which yielded good agreement with observed results in quartz sand column experiments (*54*).

When the collector efficiency η_0 is available, the attachment efficiency α can be estimated via Equation (6) (*54*):

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$$\alpha = -\frac{4r_C}{3(1-\epsilon)\eta_0 L}\ln\left(\frac{C}{C_0}\right) \quad (6)$$

where r_C is the radius of a spherical collector, ϵ is the porosity of the porous medium, L is the length of the porous medium, and C and C_0 are the particle concentrations in the column effluent and influent, respectively.

Based on the equations above, Liu et al. (*54*) estimated attachment efficiency factors for acid-treated MWCNTs passing through columns packed with quartz sand or glass beads with 8 mM Na^+ under different flow rates (0.42, 4.0, 21 and 43 m/day). The resulting α values were relatively constant (~0.14) for all experimental conditions, but this value was more than one order of magnitude greater than the theoretical value of 0.009 calculated from DLVO theory (*54*). Such a discrepancy suggests that there are factors that may impact MWCNT deposition or transport processes that had not been accounted for in the modeling. Patch-wise surface charge heterogeneity of the sand grains is likely to contribute to such deviation from classical DLVO theory (*54*).

5. Transformation

Possible CNP transformation in natural or engineered systems can change the properties of CNPs and consequently affect their mobility and bioavailability. Transformation of CNPs under natural conditions has not been fully investigated. There are, however, investigations regarding CNP reactions in chemistry and chemical engineering studies, and this information may suggest likely routes of CNP transformation in natural environments. In general, there are three types of transformations that can occur to CNPs: covalent reactions, biodegradation, and reactions of surface functional groups.

5.1. Covalent Reactions

The graphene structures of CNPs, although inert in general, are still open to covalent reactions to certain extent, which is primarily driven by the enormous strain engendered by the curvature of CNTs and spherical geometry of fullerenes (*7*, *60*). For an sp2-hybridized (trigonal) carbon atom, planarity is strongly preferred, described by a so-called pyramidalization angle of $\theta_p = 0°$ (Figure 5); whereas an sp3-hybridized (tetrahedral) carbon atom requires $\theta_p = 19.5°$ (*60*). According to the geometry of C_{60}, all of the sp2-hybridized carbon atoms have $\theta_p = 11.6°$, which is closer to the tetrahedral structure. Thus, the conversion of sp2- to sp3-hybridization can release the strain and mitigate the strain of the rest of the 59 atoms (*60*), which is consequently favorable to covalent addition (*60*, *61*). The end caps of nanotubes, if not closed by the catalyst particle, tend to be composed of highly curved (and hence unstable) fullerene-like hemispheres that are much more reactive than the sidewalls (*7*, *62*). The reactivity of these end caps is similar to fullerene, depending on the degree of pyramidalization. The

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strain energy of pyramidalization is roughly proportional to θ_p^{2} (*7*). Thus, the smaller the diameter of fullerenes or fullerene end caps, the larger the curvature is and consequently the more reactive it is. The reactivity of CNT sidewalls comes from the curvature-induced pyramidalization, analogous to but weaker than fullerene. In addition, misalignment of the pi-orbitals between adjacent pairs of conjugated carbon atoms would also contribute to CNT side-wall reactivity (*63*), for which calculations of torsional strain energies in conjugated organic molecules has provided some theoretical support (*7*, *64*).

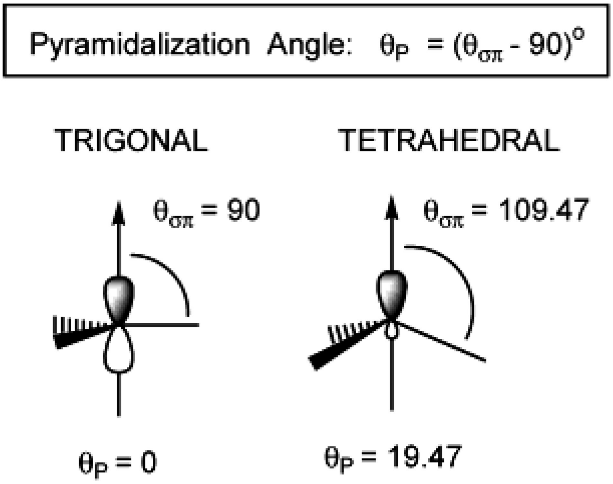
Pyramidalization Angle: $\theta_P = (\theta_{\sigma\pi} - 90)^\circ$
TRIGONAL
TETRAHEDRAL
$\theta_{\sigma\pi} = 90$
$\theta_{\sigma\pi} = 109.47$
$\theta_P = 0$
$\theta_P = 19.47$

Figure 5. Pyramidalization angle. Used with permission from Niyogi et al. (7).

Oxidization is a common form of covalent reactions occurring to CNPs and has been studied the most. Certain treatments under harsh conditions can even destroy CNPs (*65*), however, these oxidation conditions are not likely to appear in surface earth processes or in manufacturing systems. Some oxidization processes are commonly employed in CNP purification to remove impurities, and these processes generally lead to reduction of CNP sizes and addition of oxygen functional groups to CNP surfaces, changes that tend to enhance CNP mobility and perhaps bioavailability and toxicity.

5.1.1. CNT Oxidization

The unit backbone structure of CNT sidewalls, a six-numbered conjugated SP2 carbon ring, is relatively inert to oxidation. However, the sidewalls contain defect sites such as pentagon-heptagon pairs called Stone-Wales defects, sp3-hybridized defects, and vacancies in the nanotube lattice (*62*, *66*). The end caps and the defects on sidewalls are expected to be sites more susceptible to oxidization (*7*, *21*).

Major types of oxidants include strong acids, e.g., concentrated HNO_3 (*21*, *67*), mixtures of concentrated HNO_3/H_2SO_4 (*20*, *21*), $KMnO_4/ H_2SO_4$ (*21*, *68*),

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$K_2Cr_2O_7$/ H_2SO_4 (*68*), OsO_4 (*7*, *68*), and H_2O_2/H_2SO_4 (*21*), as well as strong energy inputs such as ultrasonication (*20*, *64*). However, these strong oxidative processes are unlikely to occur naturally in the environment. Oxidative reagents used in wastewater treatments, e.g., ozone (*69*), Fenton's reagent (*21*, *70*) and photophenton reagent (*71*), are also effective in oxidizing CNTs. Photooxidation is one process that can possibly occur to CNTs in the environment. An study has reported that reactive oxygen species (ROS), such as $^1O_2$, $O_2^{\bullet-}$, and $^{\bullet}OH$, were produced in carboxylated SWCNT solutions when exposed to the sunlight or lamp light within the solar spectrum, and these radicals oxidized CNTs and modified their surfaces (*72*).

The common results of oxidation are to open the end cap and introduce oxygen-containing surface functional groups like carboxyl, hydroxyl, carbonyl, and ester to attach on either the ends or the sidewalls of CNTs (*8*, *15*, *21*, *71*), although minor differences exist with various treatments. For example, Fenton's reagent (Fe^{2+}- H_2O_2) is effective in introducing both carboxyl and hydroxyl groups, while Photophenton and UV/H_2O_2 processes mostly produce hydroxyl groups (*21*). The strong oxidation processes were also found to disrupt the aromatic ring system of CNTs (*21*), for example, sonicated MWCNTs were shorter and exhibited a narrower length distribution (*27*). The consequence of these modifications is to increase the surface charge and thus stability in water.

In addition to adding surface functional groups, the oxidative treatment of fullerene-like caps and graphene layers generated oxidized polycyclic aromatic substances, which were like fulvic acids and remained sorbed on MWCNT surfaces in acidic and neutral solutions (*21*). As mentioned before, these sorbed organic matters can also help to stabilize MWCNTs in water.

5.1.2. Fullerene Oxidation

As discussed above, fullerene is generally more reactive than CNTs. The oxidization of fullerene does not require overly strong oxidants; instead, mild conditions can result in C_{60} oxidization. Prolonged mixing in water can cause negative charge and hydration on fullerene cluster surfaces (*21*, *37*, *73*). In dilute aqueous solution, the hydroxylated fullerenes, i.e. fullerenol, can be extensively mineralized by simulated solar radiation (*74*). This mineralization can reach up to 28% or approximately 47% (*74*), which is pH- and oxygen-dependent (*74*). The pH dependence of the direct and sensitized photoreactions is attributed to changes in intramolecular hemiketal formation in fullerenol (*74*). In contrast, the nC_{60} clusters formed in water are less reactive than fullerenol. Under 254 nm UV light and simulated or natural sunlight, mineralization of nC_{60} clusters was not observed, but oxygen-containing groups like epoxides and ethers were introduced (*75*). Oxygen is necessary for these oxidative transformations (*74–76*), and reactive oxidative species have been detected in these systems, including superoxide ions and singlet oxygen ($^1O_2$) (*74*).The resulting products tend to have a weaker antibacterial effect than the parent nC_{60} (*75*).

A variety of covalent reactions have been designed as modification techniques to increase the solubility of CNPs in water or organic solvents. These reactions

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include carbene chemistry (*15*, *77*), nitrene addition (*15*, *77*), hydrogenation via Birch reduction (*15*, *77*, *78*), fluorination (*79*), alkylation (*80*), arylation (*81*), and 1,3-dipolar cycloaddition (*82*). These original works have been summarized in the reviews by Niyogi et al. (*7*) and Banerjee et al. (*8*) for SWCNTs, and by Diederich et al. (*83*) for fullerenes.

5.2. Biodegradation

Recent studies indicate that carboxylated SWCNTs can be transformed via mediation by typical soil enzymes such as horseradish peroxidase (HRP), but such transformation did not seem to occur to pristine SWCNTs (*2*, *84*). During such transformations, SWCNT lengths were shortened, carboxyl groups were added to SWCNT surfaces, and CO_2 was produced (*2*). The products of the enzymatic degradation were identified as shown in Figure 6 (*2*). Recently, the same group has found that the neutrophil myeloperoxidase, a peroxidase generated inside human cells, can degrade SWCNTs and the resulting nanotubes did not generate an inflammatory response when aspirated into the lungs of mice (*85*).

Using ^{13}C-labeling, Schreiner et al. (*1*) found fullerol, the hydroxylized derivative of C_{60}, can be mineralized to CO_2 in the presence of white rot fungi after 32 weeks of incubation. Additionally, the fungi can incorporate minor amounts of the fullerol carbon into their lipid biomass, indicating the microbial utilization of fullerene derivatives (*1*). Since fullerol can be easily produced by mixing fullerene C_{60} with water, this biodegradation is thus quite probable when C_{60} enters natural systems. Figure 7 illustrates some potential environmental fates of fullerenes.

5.3. Reactions on Surface Functional Groups

There have been a variety of studies in chemistry to design reactions targeting CNP surface functional groups, carboxyl in particular, to tether additional moieties, including small molecules, macromolecules or even other particles, to further modify the CNPs for various application purposes. Figure 8 presents some of such reactions to the surface functional groups on CNPs.

6. Environmental Implications

Most studies on CNP aggregation focused on homoaggregation, i.e., aggregation among CNPs, whereas in natural aquatic systems heteroaggregation between CNPs and natural occurring colloids is more likely to dominate, because natural colloids would largely exceed the amount of CNPs (*41*). The collision rate for perikinetic aggregation and differential settling is lower when particles are of the same size; hence a monodispersed dispersion tends to be more stable than polydispersed dispersions (*6*). This implies that in natural water where other colloids or microbes are present, CNP aggregation tendency may be stronger, thus increasing the possibility of CNPs residing in the solid phase (soil/sediments).

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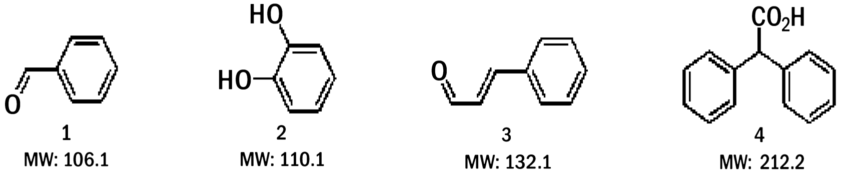
HO
HO
O
O
CO2H
1
MW: 106.1
2
MW: 110.1
3
MW: 132.1
4
MW: 212.2

Figure 6. Products identified by LC-MS for HRP-mediated degradation of SWCNTs, including oxidized PAHs such as benzaldehyde (1), 1,2-benzenediol (2), cinnamaldehyde (3), and diphenylacetic acid (4). Used with permission (2).

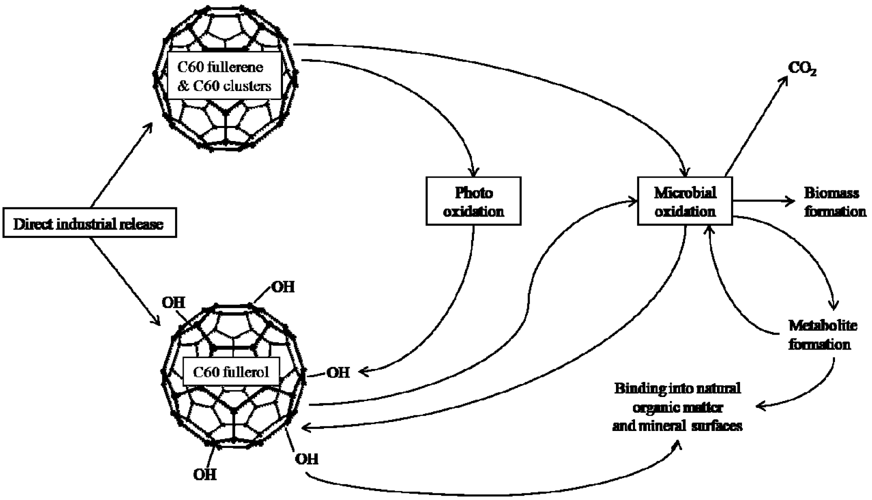
C60 fullerene & C60 clusters
Direct industrial release
Photo oxidation
Microbial oxidation
CO2
Biomass formation
Metabolite formation
Binding into natural organic matter and mineral surfaces
OH
OH
C60 fullerol
OH
OH
OH

Figure 7. Overview of the potential environmental fates of fullerenes. Used with permission (1).

Aggregation, sorption and transport of CNPs is governed by the interaction of a number of factors, including CNP surface chemistry, aquatic conditions such as ionic strength, pH, NOM concentration, and solid phase properties. Ionic strengths typical to many natural waters tend to favor deposition and thus reduce the potential exposure of CNPs. Without NOM present in water, the stable suspension of CNPs can be easily eliminated by divalent cations at low concentrations. However, NOM was found to counteract the effect of cations and dramatically stabilize CNPs at environmentally relevant concentrations (e.g., 5 mg/L). The stabilization effect of NOM is of paramount significance with regard to the potential mobility and exposure of CNPs in natural aquatic systems. Such stabilization effects enable CNPs to be transported through a longer distance and spread in a wider range. There are, however, studies showing that nC_{60} stabilized by dissolved humic substances lost the toxicity typically associated with nC_{60} when the humic acid concentrations were as low as 0.05 mg/L (*39*). The presence of NOM thus could have strong impacts on both the mobility and toxicity of CNPs, the two factors determining the potential environmental risks. Wastewater

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treatments are an important defensive line protecting potable water. Researchers found that colloidal nC_{60} aggregates and MWCNTs can be efficiently removed by a series of coagulants (e.g., alum coagulant), and in the processes of flocculation, sedimentation and filtration, while the efficiency of removal was dependent on various parameters such as pH, alkalinity, NOM contents and coagulant dosage (*51*, *86*).

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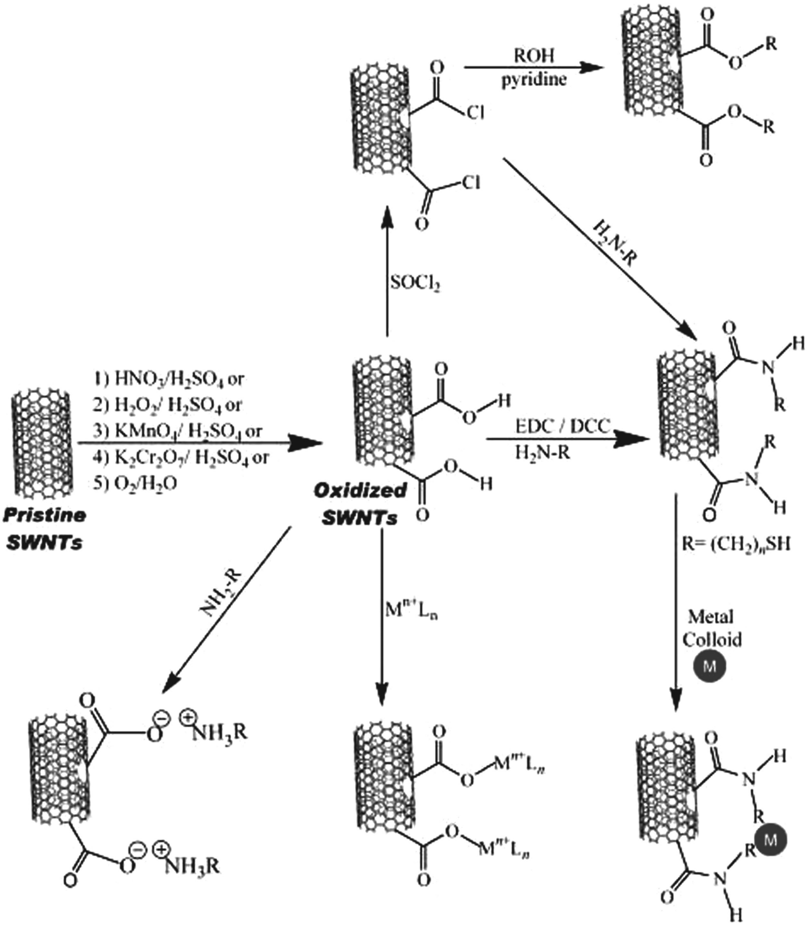
ROH
pyridine
Cl
SOCl$_2$
H$_2$N-R
1) HNO$_3$/H$_2$SO$_4$ or
2) H$_2$O$_2$/ H$_2$SO$_4$ or
3) KMnO$_4$/ H$_2$SO$_4$ or
4) K$_2$Cr$_2$O$_7$/ H$_2$SO$_4$ or
5) O$_2$/H$_2$O
Pristine SWNTs
Oxidized SWNTs
EDC / DCC
H$_2$N-R
R= (CH$_2$)$_n$SH
Metal Colloid
M
NH$_2$-R
M^{n+}L$_n$
NH$_3$R
M^{n+}L$_n$

Figure 8. Schematic of common functionalization routes used to derivatize SWCNTs at ends and defect sites. Used with permission (8).

The transformation occurring to CNPs in natural environments tends to reduce nanoparticle sizes and add on hydrophilic groups. Such changes can lead to greater CNP mobility and perhaps greater bioavailability and toxicity as well. Templeton et al. (*87*) found smaller, more mobile fractions of SWCNTs were more toxic towards an estuarine copepod than the larger fractions. Lovern and Klaper (*88*) found a similar inverse relationship between the aggregate particle size and toxicity in *Daphnia magna* exposed to fullerenes. However, after phototransformation, the toxicity of nC_{60} derivatives seemed to be less than the pristine nC_{60} (*75*). It is thus important when assessing the long-term environmental risks of CNPs to take into account potential transformation of CNPs in environmental systems.

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Chapter 5

Ecotoxicity of Fullerenes and Carbon Nanotubes: A Critical Review of Evidence for Nano-Size Effects

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The promise of nanotechnology is expected to impact almost every field with widespread incorporation of nanoparticles (NPs) in numerous commercial products. While the unique properties of NPs and their applications offer important benefits, some concerns have been raised such as the potential for NPs to pose unique risks to human and environmental health upon release into the environment. Initial speculation of novel toxicities from NPs needs to be reevaluated based on actual evidence from ecotoxicological exposure studies. In this chapter, we review the literature on ecotoxicity of fullerenes (C_{60}) and carbon nanotubes in multi-cellular organisms and evaluate the evidence for toxicological effects to be a consequence of the nano-size of these NPs. We find that absorption of these NPs and their entrance into systemic circulation has not been observed in the few studies that have investigated biodistribution in organisms under environmentally relevant conditions, and where tissue concentrations have been determined, they are exceedingly low. Limited absorption of these NPs into organisms suggests that toxicological effects reported in internal tissues should be interpreted cautiously

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and not presumed to be a nano-size effect from these NPs. Experimental artifacts such as the use of vehicle solvents (e.g., tetrahydrofuran) appear to account for the majority of the highly toxic effects observed for fullerenes. At the present time, there is not sufficient evidence to conclude a nano-size toxicological effect for C_{60} within the ecotoxicology literature for multi-cellular organisms, but there are some effects from carbon nanotubes that may be attributable to nano-size effects.

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Introduction

Nanotechnology has been described as a scientific revolution with future applications expected to transform a broad range of fields. The small particle size of nanoparticles (NPs), which are defined as having one dimension within the range of 1 to 100 nanometers, often yields exciting new properties that substantially differ from bulk particles of a similar chemical composition. With NPs already being incorporated into numerous consumer products and many more usages expected in upcoming years (for the full current list of consumer products containing engineered nanomaterials (ENs), it is suggested that the reader visit the following Web site: http://www.nanotechproject.org/inventories/consumer/), one major concern is to what extent NPs may pose environmental or human health risks (*1–3*). New technologies often have unexpected consequences and the nanotechnology revolution is not expected to be an exception. However, what is largely different about nanotechnology is that proactive research is being conducted to assess their potential risks, if any, *a priori*.

It has been postulated that nanoparticles will likely cause elevated risks as a result of their small size (*2*), which is often referred to as the so-called “nano” effect. The likelihood for heightened risks from these materials has been hypothesized to stem from their increased surface area and reactivity and the higher particle numbers for a similar mass when compared to bulk materials. While preventing unexpected risks to humans and ecological receptors is an important motivating factor for studies about the potential risks of nanoparticles, there are also serious risks to overstating results or speculating about the risks of nanoparticles without sufficient scientific evidence. Results indicating toxic effects from nanomaterials may spread fears about nanotechnology throughout the public, and later scientific evidence may not be able to sway these initial opinions. While a precautionary principle is prudent regarding toxicity of nanomaterials, overstated or unrealistic results of toxicity for a particular NP could generate negative perceptions of the nanotechnology industry and limit future benefits (*4*). This would certainly have a chilling effect on technological advancements related to nanotechnology, advancements which could have otherwise led to a substantial positive impact on our standard of living. On the other hand, it is cavalier to assume that there will be no risks from NP exposure or release into the environment, and the development of nanotechnology without consideration of the potential harmful effects could have serious negative impacts on human and environmental well-being. There is a need to understand and avoid the potential risks associated with nanotechnology, and, at the same time, to avoid overstatement of such risks that could impede realization of the full benefits of the technology. Such a balance requires the usage of environmentally realistic exposure conditions to build a solid scientific foundation related to the environmental behaviors and risks of NPs.

The extent to which early predictions about elevated "nano" risks to organisms have held true was recently examined for metal NPs and it was suggested that particles larger than 30 nm typically do not have a different toxic effect compared to bulk particles (*5*). However, this trend has not yet been evaluated, to our knowledge, for carbon nanoparticles even though some consumer goods already utilize these nanoparticles. The purpose of this chapter is to review the current scientific literature to assess to what extent the potential risks for carbon nanotubes and fullerenes, two classes of carbon nanomaterials, pose novel risks to multi-cellular organisms in the environment as a result of their nano-scale size. Methodological considerations and potential experimental artifacts unique to NPs in general and these NPs in particular will be highlighted, and an overall assessment of the evidence for nano-size effects for these materials will be provided.

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Background for Carbon Nanotubes and Fullerenes

Carbon Nanotubes

Carbon nanotubes (CNTs), first discovered by Iijima in 1991 (*6*), comprise one of the most promising classes of new materials to emerge from nanotechnology to date. Their unique structure, composed of extensive sp^2 carbons arranged in fused benzene rings, provides exceptional material properties with respect to electrical and thermal conductivity, strength, and high surface-to-mass ratios. These characteristics in turn make them suitable for numerous potential applications, including uses in composite materials, sensors, hydrogen-storage fuel cells, and various environmental applications (*7–10*). Two principal types of carbon nanotubes have been fabricated: single-walled carbon nanotubes (SWNTs), which are one-layered graphitic cylinders having diameters on the order of a few nanometers, and multi-walled carbon nanotubes (MWNTs), which comprise between 2 to 30 concentric cylinders having outer diameters commonly between 30 to 50 nm. One special type of MWNTs that has received substantial research attention is double-walled carbon nanotubes (DWNTs), which are composed of two concentric cylinders. For a more extensive discussion of the unique properties and characteristics of carbon nanotubes, please see a recent review (*10*).

Fullerenes

Carbon molecules arranged into a spherical shape resembling a geodesic dome have become known as fullerenes in honor of the visionary American architect R. Buckminster Fuller that designed prominent buildings of this configuration (*11*). Although carbon molecules can be arranged into different spherical configurations involving different numbers of carbon atoms (e.g., C_{60},

C_{70}, C_{80}, etc.), the C_{60} (buckminster fullerene or "Bucky ball") is by far the most prominent in terms of production, scientific interest, and research engagement. Considerable interest and speculation has surrounded the C_{60} fullerene since first preparation of the nanoparticle was achieved in the laboratory (*12*), and this speculation has predicted both beneficial uses (*11*, *13*, *14*) as well as unexpected negative consequences (e.g., toxicity after C_{60} release into the environment) (*1*). The elegant configuration of sixty carbon atoms into a spherical arrangement confers unique physicochemical properties to C_{60}, which have been reviewed in detail in numerous publications (*15–17*). Partially de-localized π-electrons in C_{60} can absorb energy (e.g., light) and can promote formation of reactive oxygen species (ROS) (*18*). The potential for large increases in production of C_{60} (e.g., for use in consumer products), consequent releases into the environment, and possible C_{60}-induced toxicity in organisms including humans has led to numerous recent research investigations into the environmental implications of this nanoparticle (*19*). However, studies of the environmental fate and toxicity of C_{60} are limited by a lack of established scientific methods for evaluation of the behavior of C_{60} in environmental media and for testing toxicity of C_{60} in an environmentally relevant context.

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Investigations of the Toxicity of Carbon Nanotubes

This section is divided roughly into terrestrial, sediment, and aquatic (i.e., no sediment) ecosystems. This review is intended to provide a brief overview of the literature to assess to what extent nano-size toxic effects have been observed for carbon nanotubes.

Sediment

The toxicity and bioaccumulation of carbon nanotubes in sediment ecosystems has been investigated in six studies (*20–25*). In the four studies that investigated to what extent single- or multi-walled carbon nanotubes would accumulate in organisms, researchers found negligible absorption into the organism tissues of oligochaetes (*24*, *25*), two estuarine invertebrates (*20*), and a lugworm (*21*). Changing the properties of the carbon nanotubes so that they possess higher octanol-water distribution coefficients, a change that typically corresponds with higher organism accumulation for hydrophobic organic chemicals (HOCs), was not found to increase their bioaccumulation factor (BAF) values for the oligochaete *Lumbriculus variegatus* (*25*). Given the lack of absorption into organism tissues, it is important to differentiate between carbon nanotubes inside the gut tract of the organism and those absorbed into systemic circulation. The term accumulation is hereafter used to refer to the total mass of the carbon nanoparticle in the organism while uptake specifically refers to absorption across epithelial membranes and into internal tissues. The presence of SWNTs at a high sediment concentration of 5 mg/g was found to not change or decrease the uptake of a broad number of HOCs by two estuarine invertebrates (*20*). These results agree with modeling by Koelmans and coworkers who estimated that the presence of environmentally realistic concentrations of carbon nanotubes would not be expected to impact bioavailability of HOCs in sediment ecosystems (*26*).

Perhaps unsurprisingly in light of the lack of accumulation of nanotubes by organisms, sediments spiked with carbon nanotubes typically only had a minor ecotoxicological impact even when spiked at high concentrations. MWNTs and SWNTs spiked to sediments at concentrations of 0.3 and 0.03 g/kg did not cause increased oligochaete mortality after 28 days compared to control sediments (*24*). Similarly, spiking sediment with SWNTs at a concentration of 0.03 g/kg did not impact burrowing behavior, feeding rates, or DNA damage as measured by the comet assay for a lugworm (*21*). However, decreased survival was observed for *Hyallela azteca* and *Leptocheirus plumulosus* albeit at environmentally unrealistic concentrations of 300 g MWNT/kg sediment and 30 g/kg, respectively (*22*). While spiking MWNTs at a concentration of 99 g/kg did not have an impact on *H. azteca*, the lowest observed effect concentration was not investigated for *L. plumulosus*. It was also observed that the LC50 (i.e., concentration that is lethal to 50 % of the organisms) value for raw MWNTs using these same organisms was higher than that for activated carbon and carbon black (*23*). This suggests that MWNT toxicity may be less than that for activated carbon, an amendment that is being widely considered for treatment of contaminated sediments (*27*, *28*). Overall, the accumulation and toxicological results reported to date for sediment ecosystems do not indicate that these carbon nanotubes possess a uniquely elevated risk as a result of their nano-scale size.

Soil

There have been several studies on the effects of carbon nanotubes in earthworms (*25*, *29–31*). SWNTs and MWNTs did not accumulate within earthworms to significant extents even when the MWNTs were modified to be more hydrophilic (*25*, *29*). Additionally, both SWNTs and MWNTs were found to decrease earthworm uptake of pyrene, a polycyclic aromatic hydrocarbon, when the nanotubes were spiked to soils at concentrations of 3 g/kg, but not 0.3 g/kg (*30*). When considered in combination with prior experimental (*20*) and modeling (*26*) efforts, these results suggests that the presence of carbon nanotubes in the environment are expected to decrease HOC accumulation by organisms in a manner similar to that for black carbons when added at extremely high concentrations. The toxicity of DWNTs was also investigated using the earthworm *Eisenia veneta* (*31*). The most sensitive endpoint was reproduction as measured by earthworm cocoon production which was impacted at a food concentration above 37 mg DWNT/kg food, while survival and hatchability were not impacted at concentrations up to 495 mg DWNT/kg food. These results are not believed to result from metal catalysts associated with the DWNTs, but the toxicity mechanism was not determined. In a study by Petersen and coworkers (*30*), no effects were observed on the earthworm lipid content or dry mass after exposure to concentrations up to 3 g/kg for SWNTs and MWNTs in two soils, although this study was not specifically designed to test for subacute toxicity effects. Nevertheless, these results suggest that carbon nanotubes may cause

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sub-acute effects to organisms such as impacting their reproduction behaviors at relatively high concentrations, but that it is highly unlikely for them to impact the survival rates for adult organisms.

There have also been numerous studies on the effects of CNTs on plants conducted under hydroponic conditions (i.e., without soil). The effects of interactions with soil were not considered in any of these studies, although they would likely decrease the observed toxicity as a result of sorption/attachment interactions. Nevertheless, the observed effects of CNT exposure in plant species have been inconsistent. One study indicated that MWNT treatment did not impact plant germination for any of the plant species tested at an MWNT solution concentration of 2 g/L (*32*), while other studies showed decreases in root elongation for some plant species and increases for others after exposure to functionalized and non-functionalized SWNTs (*33*) or decreased biomass for *Cucurbita pepo* (zucchini) after MWNT exposure (*34*). The toxic effects observed for the zucchini appeared to be related to the properties of the dispersed carbon nanotubes, because activated carbon did not have this effect. Thus, this may be an effect related to the nano-sized structure of the carbon nanotubes. CNTs generally had a more pronounced effect on suspended plant cells (*32*, *35*) with MWNTs causing decreased cell viability and increasing reactive oxygen species at a concentration of 20 mg CNTs/L of medium (*35*). Carbon nanotubes showed an ability to pierce plant cells *in vivo* using two photon microscopy (*36*) but they did not fully enter the cells, and SWNTs did not appear to enter the roots of any plants when investigated by scanning electron microscopy (SEM) (*33*). These results agree with transmission electron microscope (TEM) micrographs of suspended rice cells exposed to MWNTs which showed MWNT contact with the cell wall but not internalization of the nanotubes (*35*). Raman spectroscopy showed uptake of MWNTs into tomato plant seeds, but they were not detectable in grown plant tissues (roots, leaves, or stems) (*37*). Overall, these results suggest that CNT internalization by plants will be limited. Additionally, some studies have indicated that carbon nanotubes had a positive effect on tomato plants enhancing germination rates and shortening the germination time (*37*), and non-functionalized SWNTs enhanced root elongation in onions and cucumbers (*33*). As such, the expected effects of carbon nanotubes on plant growth in hydroponic conditions are unclear and may vary based on the type of nanotube and plant species, yet some of these observed effects may be a result of the nano-sized structure of the CNTs. However, it is important to recognize that these effects may substantially differ in the presence of soil as would be typical for plant exposure in the natural environment. At a minimum, extreme caution is warranted in the usage of carbon nanotubes for agricultural products given the lack of a rigorous understanding about the risks these NPs could pose after ingestion.

Water

The majority of studies relating to the ecological impacts of carbon nanotubes have been conducted in water-only exposures. This may be in large part a result of the fact that detection of carbon nanotubes in matrices without soil or sediment is substantially easier. Unlike the studies conducted in soils and

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sediments, suspended carbon nanotubes have shown acute and sub-acute effects to organisms at low concentrations in the range of micrograms of nanotubes per liter of solution (i.e., (*38*)). Their toxicity has been investigated using a broad range of ecological organisms including fish (*39–42*), daphnia (*22*, *23*, *38*, *43–45*), estuarine copepods (*46*), amphibian larvae (*47*, *48*), protozoa (*49*), and bacteria (*50–53*). Despite the observed toxicity to these various organisms, there did not appear to be substantial absorption of MWNTs across the intestines by *Daphnia magna*, but rather a large mass of MWNTs appeared to be compacted in the organisms guts as measured microscopically and using radioactively labeled carbon nanotubes (*44*). Large masses of carbon nanotubes were also found in the guts in many other organisms using microscopic methods (*23*, *45*, *47*), but no paper to our knowledge has shown substantial absorption of carbon nanotubes across the gut linings in any aquatic organism.

As such, toxic effects from CNT exposure are expected to occur primarily in the digestive organs or gills, or after attachment to the surfaces of organisms, which could potentially influence their ability to swim as has been observed earlier for *Daphnia magna* exposed to fullerenes (*54*). Indeed, two studies with lipid-coated SWNTs and daphnia have suggested that the observed toxicity was likely a result of clumping and deposition in the organism intestines (*43*, *45*), which thus raises the question about whether the effects observed for NPs were a result of a nano-size effect or just suspended solid material that could deposit in the gut. Additionally, Kennedy and coworkers found that stirred MWNTs, which were more aggregated than sonicated MWNTs, were more toxic to *Ceriodaphnia dubia* than for sonicated nanotubes from the same source (*22*), a result which again contrasts with what would be expected for a nano-size toxic effect. One potential artifact which could be the cause of toxicity in studies with carbon nanotubes is the release of toxic metals from the catalyst materials. It was recently determined that yttrium released from carbon nanotubes affected the functioning of neuronal calcium channels (*55*). The impact of released metals was also suggested as a potential cause of the differing effects of SWNT and DWNT exposure on zebrafish embryos (*41*). It is important to note that broad differences were observed in the toxic impact of the CNTs on the various organisms. Larvae of the amphibian *Ambystoma mexicanum* did not exhibit increased mortality or genotoxicity after exposure to DWNTs at concentrations up to 1 g/L (*48*), while *Daphnia magna* had a 96-h LC50 value of 2.48 mg MWNTs/L (*38*). The cause for the substantially different sensitivities among these organisms to carbon nanotube exposure is a topic for future research.

Investigations of *O. mykiss* exposed to SWNT indicated some lesions in the brains (*42*), an effect that would raise serious concerns about the ecotoxicological effects of these materials if they were indeed determined to be the cause of such effects. Smith and coworkers (*42*) exposed *O. mykiss* to SWNTs (0.1 to 0.5 mg/L) for 10 days and found altered trace metal concentrations, specifically elevated Cu and Zn, in the brain. *O. mykiss* were also more aggressive, had higher ventilation rates, and poorer buoyancy control compared to control fish indicative of alterations in behavior. Similar behavioral changes have been observed as a result of fish exposure to waterborne pollutants which may be linked to underlying alterations in brain functioning (*56*). However, both effects on behavior and

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trace metal homeostasis in trout exposed to SWNTs were also evident in solvent controls and the overall contribution of SWNT to these toxicities is uncertain. Brain pathologies were, however, not related to solvent exposure. Necrotic cell bodies and small foci of vacuoles were evident to varying extents in brains from all SWNT exposed fish, and swelling of blood vessels on the ventral surface of the cerebellum was observed and suggestive of vascular injury in these fish. Enlarged blood vessels could be due to hyperaemia as a result of respiratory distress generated by occlusion of gills by the accumulated SWNTs. Histological changes in the brains of *O. mykiss* were not detected after 6-week dietary SWNT exposure (500 mg/kg food) (*39*) and whether lesions in the brain reported in Smith and coworkers were mediated by toxicities at the gill or absorption of SWNT from the aqueous phase is unclear.

There are, however, some impacts that appear to be nano-size effects or are related to the intrinsic properties of the CNTs. For example, derivitization of the carbon nanotubes to give them various functional groups dramatically impacted their toxicity to *Ceriodaphnia dubia* with positively charged functional groups increasing acute toxicity and hydrophilic functional groups eliminating it (*22*). Carbon nanotubes with specific functional groups that enhance the nanotube's toxicity may indeed cause elevated risks if they are present in water bodies at sufficiently high concentrations, and thus risk assessment for carbon nanotubes should take into consideration the functional groups on the carbon nanotubes. In a separate study, small fluorescent nanocarbon byproducts were shown to increase life-time mortality of estuarine copepod *Amphiascus tenuiremis* at a concentration of 10 mg/L (*46*), while purified SWNTs did not have an effect at this concentration. This nanocarbon fraction had average lengths less than 18 nm and widths and heights near 1 nm and were thus much smaller than the purified SWNTs. Investigating whether similarly small SWNTs would have elevated toxicity is a topic for future research.

One major concern for the environmental relevance of these experiments is the extent to which carbon nanotubes would remain suspended in aquatic ecosystems, or whether the nanotubes would rapidly form aggregates and settle out of the solution. This issue will not be discussed at length in this chapter, but it has been studied extensively (*57–61*), and natural organic matter appears to be one of the primary influential factors (*39*). Additionally, the relatively low CNT concentrations tested for the water-only exposures are still orders of magnitude larger than those average concentrations estimated to be found in the water phase by modeling (*62*). Lastly, most studies use sonication to suspend the carbon nanotubes in solution, but it is unclear to what extent carbon nanotubes would be similarly well dispersed in ecological systems and whether this process overestimate the capacity for carbon nanotubes to remain suspended in the natural environment thereby potentially overestimating their likely risks.

Investigations of the Ecotoxicity of C_{60} Fullerenes

The toxicity of C_{60} has been investigated in ecotoxicity tests and results have been reported in the literature, which provide some initial information (e.g., LC_{50}

values) to consider for environmental risk assessments. However, there have been significant technical limitations within the emerging discipline of nanotoxicology, and toxicity of C_{60} reported in previous studies must be critically evaluated to determine if any conclusions can be drawn regarding the toxicity of this NP. The objective of this review is to critically examine some of the previous ecotoxicity research and to assess the weight of evidence for a nano-size effect attributable to C_{60} exposure. Although we are aware of the literature emerging on effects of NPs in microorganisms, this review will focus on studies that have investigated multi-cellular organisms rather than unicellular organisms

Natural production of C_{60} has occurred on earth for as long as combustion of carbon proceeded in the absence of oxygen and evidence indicates that forest fires, volcanic eruptions, and meteoritic impacts can all generate C_{60} (*63*). The issue for environmental nanoscience is whether anthropogenic production of C_{60} will generate significant additional quantities of C_{60}, and if this C_{60} will be released in a manner that will generate excessive exposure in biological receptors to cause negative biological effects. Currently, use of C_{60} in consumer products is limited to a few personal care products (e.g. skin creams, see http://www.nanotechproject.org/inventories/consumer/) and estimates of annual releases of fullerenes to various environmental compartments for the US and Europe are available (*62*). Future applications of C_{60} that will increase releases of C_{60} into the environment should be considered; however, the types of products that use C_{60} will inform on routes of disposal and some routes of disposal are not likely to increase environmental concentrations of C_{60} appreciably. For example, use of C_{60} in consumer products will likely lead to disposal through wastewater or as solid waste, and ultimate removal by incineration or burial in landfills-both projected to lead to very little release of C_{60} into the environment (*62*). Some release of C_{60} into surface waters could occur in effluents from wastewater treatment plants or perhaps if C_{60} is used in the future for environmental remediation projects, and, in either case, understanding the ecotoxicity of C_{60} in soils or in surface waters will be important.

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Issues Regarding Toxicity of C_{60}

Numerous articles have hypothesized that toxicity of C_{60} is a consequence of oxidative stress (e.g., review (*19*)) and this hypothesis is consistent with the ability of C_{60} to generate ROS under specific conditions (*64*, *65*). Generation of ROS is clearly a consequence of the nanoscale characteristics of this NP, and, if oxidative stress occurs in organisms exposed to C_{60}, then this could be considered evidence of a nano-size effect attributable to this NP. However, the ability of C_{60} to generate ROS has been reported to be limited to when the NP exists as an individual fullerene (i.e., C_{60} dissolved within a solvent) rather than within an aggregate of nC_{60} in the aqueous phase (*66*). If C_{60} does generate ROS when organisms are exposed, oxidative stress can be expected with consequent effects on biological processes.

A central question regarding the toxicology of C_{60} is whether uptake and distribution of the NP is required for toxicity to occur or whether toxic effects (e.g., ROS) can be exerted without transport of C_{60} across epithelial membranes.

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Evidence for uptake of C_{60} across cell membranes is limited, and the most convincing cases have been investigations of pulmonary toxicity in which rodent models have been exposed to nC_{60}. Macrophages were found to contain C_{60} after exposure and appeared to be involved in clearance of the NPs from alveolar surfaces. When nC_{60} has been injected (intraperitoneal (ip)) into rats, transport to tissues was reported and accumulation appeared in the liver, kidney, and spleen occurred as would be expected from the acute (up to 1000 mg/kg) doses administered (*67*). Uptake after oral administration in rats indicated that water soluble $^{14}C_{60}$ (generated by preparation of C_{60} in saline containing 0.2 % Tween 80) was minimal and that the administered C_{60} was voided with feces (*68*). Other oral exposures of rodent models to C_{60} have reported no indications of toxicity in the exposed organisms (*67*, *69*)). No information on dermal uptake of C_{60} is available, although one study reported no skin irritation in humans exposed for 96 h with a skin patch (*70*). The accumulation of C_{60} on the surface of tissues within (e.g., on alveolar surfaces, (*71*)) or on the external surfaces has been documented in various organisms (e.g., (*72*)), but the toxicological consequences of this accumulation are uncertain. Fullerenes did not appear to be readily absorbed based on microscopic examination of microvilli by TEM and most fullerenes were present as large aggregates within the gut lumen of *Daphnia magna* (*73*). Additionally, fullerene accumulations within the gut lumen appeared to be limited by the size of the gut rather than the aqueous phase concentration, again suggesting minimal systemic absorption into the organism. It is possible that accumulation of nC_{60} could influence respiratory processes in some organisms or alter digestive system function during dietary exposure without uptake of the NPs across epithelial membranes; however, whether this would constitute a nano-size effect is questionable. Within the ecotoxicity literature there is no clear evidence of uptake of C_{60} across epithelial membranes.

Ecotoxicity of C_{60} in Aquatic Organisms

Challenges of testing toxicity of nanomaterials include careful characterizations of numerous particle-related properties (discussed in detail in (*2*)) of starting materials and accurate determination of physicochemical properties during exposure (*19*). The ability to obtain C_{60} of relatively high purity (e.g., >99.9 %) that can be generated without use of toxic catalysts (e.g., metals used in generation of CNTs, (*74*)) combined with considerable previous research on properties of C_{60} (*75*) provide a strong foundation for toxicity studies. However, the extreme insolubility of C_{60} ($<10^{-9}$ mg/L) (*76*) and tendency to form colloidal aggregates of nC_{60} (*77*) that have a strong affinity to ad/absorb substances (*78*) from the aqueous media generate scenarios that make testing toxicity difficult and limit comparability among studies. While numerous review articles demand that careful characterization of C_{60} physicochemistry be conducted during toxicity tests (e.g., (*19*)), there is not a consensus on what would constitute sufficient characterization during exposures and no reports have to our knowledge related any physicochemical property of nC_{60} to toxicity. Due to the complexity and changing physicochemistry of nC_{60} that is inherent in environmentally relevant exposures, complete understanding of nC_{60} behavior

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may be an unrealistic goal, and such an undertaking may not even be necessary if these properties are not shown to dramatically impact any toxic effects observed after organisms are exposed to fullerenes.

Generation of nC_{60} in the aqueous phase has been conducted by several techniques and each technique has limitations regarding environmental relevance and implications on toxicity assessment. C_{60} is soluble in organic solvents (*79*), and the solvent tetrahydrofuran (THF) has been found to be particularly useful to produce relatively consistently sized nC_{60} after transfer into the aqueous phase and removal of THF by evaporation (*80*). However, the configuration of nC_{60} enables retention of THF (and other solvents e.g., toluene) within C_{60} aggregates that confounds subsequent evaluations of physicochemistry and toxicity (*81*). nC_{60} can also be generated by addition of C_{60} to pure water and ultrasonication over varying periods of time; however, transmission of high energy on the nanoscale to nC_{60} can change surface chemistry and perhaps generate functionalized fullerenes with different properties (*82*). Long-term (weeks to months) stirring of C_{60} in water (both in natural light and in dark) can lead to the formation of nC_{60} in a manner considered by some to be most environmentally relevant (*72*); however, the extent of formation of hydroxylated C_{60} on the surface of nC_{60} over time is unknown as are the consequences of such changes on environmental fate and ecotoxicity.

The hypothesis that C_{60} (THF generated nC_{60}) can induce oxidative injury in aquatic organisms was supported in early studies (*83–85*) but has subsequently been refuted as techniques for investigating toxicity of C_{60} have been refined. Toxicity attributed to C_{60} in those studies is more likely linked to THF decomposition products as demonstrated in a study with zebrafish (*86*) and further confirmed in subsequent research (*87*). Results that nC_{60} (THF generated nC_{60}) does not generate oxidative injury (or any other toxic effects) when THF and THF decomposition products are removed (*88*) convincingly rejected the hypothesis that C_{60} was responsible for the toxicity reported in studies that have used THF-nC_{60}. Despite this evidence, numerous articles continue to cite studies that have used THF-C_{60} to indicate toxicity of C_{60} (e.g., (*89*)). Results of THF-nC_{60} investigations demonstrate the challenges of testing the toxicity of NPs, but are not acceptable for further discussion about the toxicity of nC_{60} (*88*).

Oxidative stress has been reported in fish exposed to nC_{60} generated by techniques other than solvent exchange and could appear to support the hypothesis that C_{60} can generate ROS and cause toxicity. In fathead minnow *Pimephales promelas*, significant induction of CYP2-like isozymes and elevated lipid peroxidation (liver, gill, brain) was reported (although data was not shown) after 48-h exposure to water stirred nC_{60} (*90*). Results from some of the same investigators as Zhu et al., (*90*) report subsequently that there was no effect of water stirred nC_{60} on CYP2-like isozymes in *P. promelas* or the Japanese medaka *Oryzias latipes* or evidence of lipid peroxidation, which led to the conclusion that traditional biomarkers of oxidative stress were not adequate to demonstrate effects of C_{60} (*91*). Chronic (32 day) exposure to water-stirred nC_{60} had subtle but significant decrease in growth of carp *Crassius auratus* and some significant changes in antioxidant enzyme activity (catalase, superoxide dismutase) in some tissues, but effects, although statistically significant, were not related to

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concentration of nC_{60} (0.04, 0.2, 1.0 mg/L) (*92*). Shinohara et al. (*93*) examined the potential for oxidative injury in common carp *Cyprinus carpio* brains to result from nC_{60} exposure and demonstrated that changes in indicators of oxidative stress were actually a consequence of the assay technique when nC_{60} is present and that if the assay was conducted under lighted conditions then oxidative stress was detected. These results could explain the inconsistencies in oxidative stress indicators reported in the study of Zhu et al., (*92*). In female *Fundulus heteroclitus*, glutathione levels were variable but significantly elevated after exposure to 2.5 and 10 mg/L water-stirred nC_{60}, but no other toxic effects were detected (*72*). Six-week dietary exposure to 500 mg C_{60}/kg food, in juvenile rainbow trout *Oncorhynchus mykiss*, did not cause any changes in oxidative stress endpoints in all major body systems considered (*39*). Overall, the link between ROS-related toxicity and exposure to C_{60} is questionable and has not been separated adequately from effects of vehicle solvents or assay techniques.

The toxicity of C_{60} has been investigated in various aquatic invertebrates and there is evidence of negative consequences of exposure. Investigations that have used nC_{60} generated by THF will not be considered further here for the reasons indicated above; however, nC_{60} produced by long-term stirring in water can affect some aquatic invertebrates. Filter feeding invertebrates can accumulate nC_{60} within their digestive tract and also nC_{60} have been described to adhere to organism surfaces (e.g., *Daphnia magna*, (*94*). Results of acute toxicity in *D. magna* indicate a lack of a dose response to nC_{60} (*85*, *95*) and inability to achieve 100 % mortality even at concentrations up to 500 mg/L (*83*). Of interest are sub-acute responses including reductions in growth (decreased molting) and reproduction in *D. magna* (*95*), and indications that the accumulation of nC_{60} within the digestive tract and on body surfaces may have caused physical disruption and perhaps limited uptake of nutrients. In eastern oysters *Crassostrea virginica* exposed to nC_{60}, toxicity was reported in development of embryo and larval forms (*96*); however, the nC_{60} was prepared by solvent exchange (solvent was toluene) and the contribution of the solvent on toxicity was not completely determined. Within the C_{60} ecotoxicity literature in invertebrates there is evidence for physical effects consequential to the accumulation of nC_{60} aggregates on tissue surfaces; however, there is no evidence for toxicity by other mechanisms (e.g., oxidative stress etc.). Physical disruption of tissue surfaces is a reasonable consequence of accumulation of nC_{60}, but does not constitute a “nano” effect. Unfortunately, controls for a particle effect (e.g., inclusion of amorphous carbon black as a treatment) have not been conducted to determine if effects of surface accumulation of nC_{60} are unique to C_{60} or a general organism response.

Ecotoxicity of C_{60} in Soils and Sediments

In the sole study of fullerene toxicity to multi-cellular soil organisms, no effects were observed on earthworm mortality, reproduction, or growth at food concentrations up to 1000 mg C_{60} per kg food (*31*). There are not yet any published studies on the ecotoxicity of fullerenes in sediment dwelling organisms. While the current results with earthworms suggest minimal C_{60} toxicity to organisms in soils,

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additional research is needed to more fully evaluate the potential risks of fullerenes in these ecosystems.

Conclusions

As the emerging nanotechnology industry matures there is an important need for guidance on the development of this technology to appropriately consider the risks posed by the intentional and unintentional release of NPs into the environment. Nanoparticles do have unique properties, and there are, therefore, risks of novel toxic effects; however, the precautionary principle must be balanced by critical evaluation of the evidence obtained from investigations of toxicity of NPs. Early speculation regarding the potential for ecotoxicity of C_{60} and CNTs was prudently based on understandings of the properties of these NPs. Now that numerous investigations on the toxicity of C_{60} and CNTs have been completed, it is appropriate to re-visit the early speculation and determine how well it is supported by experimental evidence. Evidence for a nano-size effect attributable to C_{60} has not been demonstrated when confounding factors of the experimental design and assay techniques (e.g,, vehicle solvents etc.) are controlled in ecotoxicity studies. Likewise investigations with CNTs have not consistently supported a nano-size related effect, although nano-size toxic effects may have been implicated in a small number of studies. A particular limitation in the connection between C_{60} or CNTs and toxicity in multicellular organisms is that uptake of these NPs across epithelial membranes through normal exposure routes (integument, respiratory surfaces, gastrointestinal tract) is extremely low. Toxic effects exerted on tissue surfaces have been documented but either did not include appropriate controls (e.g., amorphous carbon black) or controls indicated similar effects to NP treatments suggesting that a nano-size effect was unlikely. Not detectable, or extremely low, absorption of C_{60} and CNTs across epithelial membranes and accumulation within tissues (i.e., not accumulation within gut lumen or attached to tissue surfaces) indicates that biomagnification through the food web is not a likely scenario for these NPs after release into the environment. This review is by no means the final word on this topic as techniques for measuring toxicity of NPs and most appropriate effect endpoints to consider are likely to continue to evolve; however, continued discussion of C_{60} and CNT ecotoxicity should move forward from the evidence based on existing ecotoxicity data rather than on early speculation of potential novel toxicity from these NPs. Testing for nano-size effects should continue, but based on existing evidence, nano-size related ecotoxicological effects should not be expected for these NPs.

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Chapter 6

Nanotoxicology in the Microbial World

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Microorganisms play critical roles in the biological, geological, geochemical, and climactic balance of environmental ecosystems. Nanotechnology and its variously engineered nanomaterial products have been implicated as potentially disruptive influences on microbial community health and preservation, thus contributing towards perhaps significant and lasting ecological and evolutionary consequences. The current debatable state of nanotechnology risk assessment as it pertains to microbial ecology and ecotoxicology will be discussed in this chapter.

1. Introduction

Microorganisms are the foundation of environmental ecosystems and play key roles in primary productivity, global biogeochemical cycling, pollutant degradation, and wastewater management. Upsetting these roles can have dire consequences. The nanotechnology revolution and its nanomaterial outputs are considered a potential threat to microbial life, which here includes the prokaryotic bacteria, the bacteriophage viruses that prey on bacteria, and the lower eukaryotes consisting of the fungi (molds and yeasts), protozoa, and algae (Figure 1). Nanomaterials are discharged into the environment either unintentionally as, for example, disposed consumer products and construction materials or as industrial point source emissions, or intentionally for applications such as water treatment, soil bioremediation, and nano-scale sensors. Upon entry into aqueous, soil, or wastewater ecosystems, they are suspected of exhibiting disruptive antimicrobial effects. However, the bacteriocidal and bacteriostatic potential of nanomaterials is an intensely debated issue, with the relevant scientific literature proliferating at a rapid pace over the past decade (Figure 2). Experimental proof that nanomaterials

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kill or harm microorganisms exists, but it is highly dependent on the experimental conditions applied and critics often cite the optimization of these conditions towards cytotoxic outcomes as not necessarily true to life. This includes, for example, nanomaterial inputs exceeding anticipated environmental discharge rates and a general disregard for the myriad environmental factors that influence nanomaterial/microbial interactions (organic matter, pH, ionic strength, salinity, etc.). Predicting the ecological impacts of nanomaterials within the environmental matrix will clearly be a complicated task but remains critically necessary for the development of appropriate risk assessment models. This chapter provides a current overview of the scientific literature in support of and countering microbial nanocytotoxicology among the nanomaterial family of carbon nanotubes, C_{60}, silver, and metal oxide nanoparticles, and quantum dots and their potential influences on the global microbial biosphere.

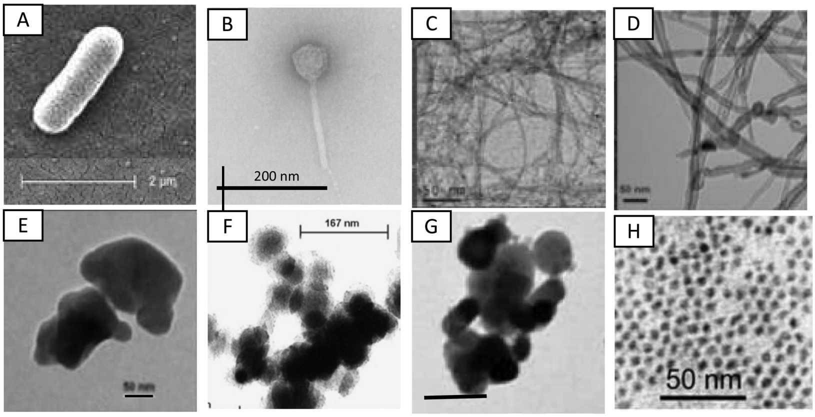

Figure 1. Representative electron micrographs of the microbial world versus the nanomaterial world (A) an Escherichia coli bacterial cell (bar = 2 µm), (B) a bacterial virus (bacteriophage) (bar = 200 nm), (C) single wall carbon nanotubes (bar = 50 nm), (D) multi-wall carbon nanotubes (bar = 50 nm), (E) nC_{60} nanoparticles (bar = 50 nm), (F) silver nanoparticles (bar = 167 nm), (G) zinc oxide (ZnO) nanoparticles (bar = 50 nm), and (H) cadmium-selenide (CdSe) quantum dots (bar = 50 nm). (Photos courtesy of the Centers for Disease Control, Van Patten Nanoscience Lab - Ohio University Kora et al. (6), Kim et al. (7), and Kang et al. (9).)

2. Carbon Nanotubes

Carbon nanotubes (CNTs) are rolled up graphene sheets comprising a hexagonal network of covalently bonded carbons. CNTs encompass a large class of materials that includes single wall CNTs (SWCNTs (or also referred to as SWNTs)), which consist of one graphene cylinder, multi-wall CNTs (MWCNTs (or MWNTs)), which have multiple concentric cylinders, or structures made of stacked cones, cups, or plates (also referred to as carbon nanofibers (CNFs)). The chemical, mechanical, and electrical properties of these nanomaterials strongly depend on the geometry of the constituent graphene layers, exposed graphene edges at the tips or side walls of the nanotubes, and defects within the graphene network. There is a growing number of works on elucidating the mechanisms of interactions of CNTs with mammalian cells and tissues driven by interest in the effects of direct exposure to humans (*8*). However, understanding CNT interactions on the microbiological scale has been much more limited. In 2007, Kang et al. (*10*) combined pristine SWCNTs (~0.9 nm diameter) at 5 µg/ml with *Escherichia coli* K12 cells (10^6-10^7 cells/ml) and demonstrated 80% reductions in cell viability after approximate one hour exposures. Scanning electron micrographs (SEM) showed significant damage to cell wall integrity and a measured increase in free nucleic acid after SWCNT exposure corroborated loss of cell membrane permeability. It was therefore hypothesized that direct contact of SWCNTs with bacterial cells was the likely mechanism responsible for cell death. They also exposed *E. coli* K12 cells (10^7 cells/ml) to larger 30 nm diameter MWCNTs at 5 µg/ml and demonstrated reduced levels of cytotoxicity (~30% loss of viability) and reduced concentrations of free nucleic acid, thereby confirming that smaller diameter SWCNTs were more toxic to bacterial cells, presumably due to more efficient piercing of the cell membrane (Figure 3) (*4*). However, longer term (48 hour) exposures of *E. coli* K12 to SWCNTs at moderate concentrations of 5-300 µg/ml did exhibit growth recovery, presumably because those cells that are killed are providing nutrients to those cells that remain alive, thus enhancing growth (*11*). In parallel, some percentage of the SWCNTs would likely become bound up and aggregated within the matrix of dead cell constituents, essentially negating their cytotoxicity. However, as SWCNT concentrations increased beyond 300 µg/ml, cytotoxicity within the bacterial population became more absolute and recovery more difficult.

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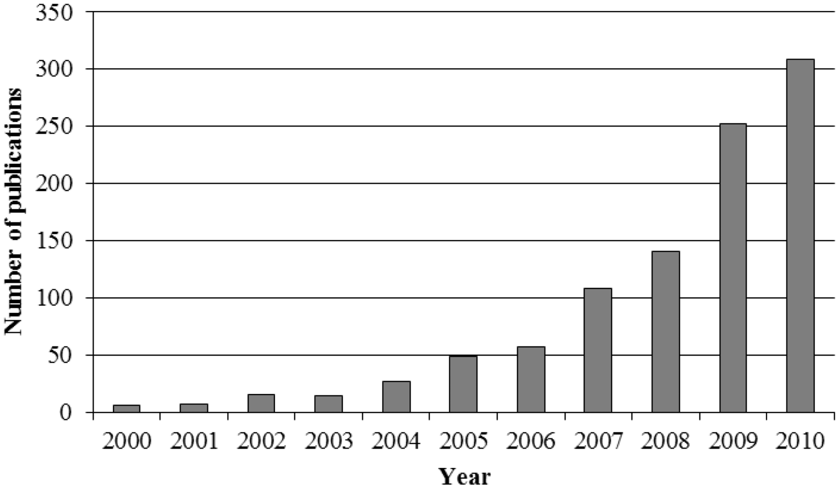

Figure 2. Publication trends over the past decade describing nanotechnology and its toxicological association with microorganisms (ISI Web of Science search using the keyword string (nanomaterial or nanoparticle* or nanotech*) and (bact* or microbio* or microorg*) and (tox*).*

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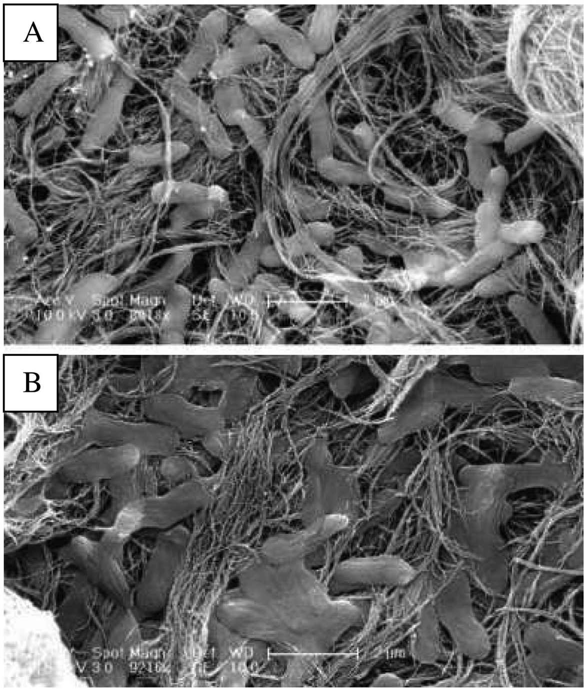

Figure 3. Scanning electron micrographs of E. coli cells after a 60 minute aqueous exposure to (A) MWCNTs and (B) SWCNTs. (Used with permission from Kang et al. (4))

Liu et al. (*12*) combined pristine SWCNTs (~0.83 nm diameter) at 5 µg/ml with both Gram negative (*E. coli* and *Pseudomonas aeruginosa*) and Gram positive (*Bacillus subtilis* and *Staphylococcus aureus*) bacterial cells (10^6-10^7 cfu/ml). Gram negative cells have a more complex outer membrane whose mechanical properties under atomic force microscopy (AFM) measurements lend more surface 'stiffness' to the cell. Thus, the piercing mechanism of SWCNTs should be more detrimental to Gram positive cells than to Gram negative cells, which indeed was the case. SWCNTs killed 47-57% of the Gram positive bacteria but only 22-38% of the Gram negative bacteria. Like the previous studies described above, these death rates occurred using aggregated suspensions of SWCNTs, and it was intuitively hypothesized that individually dispersed SWCNTs would function more effectively in killing bacterial targets. These so-called 'nano dart' fractions of SWCNTs were shown to be significantly more cytotoxic, killing 80-94% of the Gram positive bacteria and 53-67% of the Gram negative bacteria. MWCNTs were similarly shown to be more toxic when dispersivity was increased (*13*). Based on artificial piercing of bacterial cells with a 2 nm diameter AFM tip, it was concluded that single episode collisions between CNTs and cells would not result in direct physical damage (*14*). Rather, it is likely the accumulative effect of many CNT collisions and piercings that ultimately destroy the cell. Arias and Yang (*15*) performed similar SWCNT (~1.5

nm diameter; 25 to 250 μg/ml) and MWCNT (~15-30 nm diameter; 100-875 μg/ml) exposure experiments using Gram negative *Salmonella typhimurium* and Gram positive *B. subtilis* and *S. aureus* (each at ~10^7 cfu/ml). They likewise showed better antimicrobial activity with SWCNTs than with MWCNTs and demonstrated that SWCNT antimicrobial effects were highly dependent on the buffer composition (i.e., being very effective in distilled water and saline but less in phosphate buffered saline and brain heart infusion broth, possibly due to higher ionic strengths limiting the interactions between the CNTs and cells) and increased with increasing SWCNT concentration (optimal at 200-250 μg/ml).

Kang et al. (*9*) looked at SWCNT (~1.2 nm diameter) and MWCNT (~17.4 nm diameter) cytotoxicity under more real-world conditions using river water and wastewater effluent as test matrices. Water samples were passed through a filter coated with SWCNTs or MWCNTs, and the more efficient impacting of cells directly onto CNTs must be taken into consideration when comparing this study to the studies above where CNTs interacted with microbial cells freely in solution. SWCNTs performed best under these conditions with a 60-70% reduction in cell viability followed by MWCNTS that inactivated cellular constituents at 15-40%. The presence of natural organic matter (NOM), which affects CNT surface charge, aggregation characteristics, and mobility, was shown to reduce the attachment of bacteria on SWCNT aggregates by 50% but without a corresponding reduction in antibacterial effectiveness towards these attached cells. The antibacterial activity of MWCNTs in the presence of NOM has yet to be reported, but MWCNTs can become more stable in the water column due to NOM interactions, and thus exhibit a longer residency time that could theoretically equate to higher microbial cytotoxicity (*16*). Luongo et al. (*17*) assessed the effect of MWCNTs on an activated sludge microbial community and demonstrated inhibition of microbial respiration activity with dose dependent correlation to increasing MWCNT concentrations (up to 3.24 μg/ml). Goyal et al. (*18*) performed similar assessments of SWCNT cytotoxicity in activated sludge. SWCNTs at 219 μg/ml were combined with activated sludge from a municipal wastewater treatment facility. Changes in microbial community structure over a 5 hour exposure period were assessed using the molecular fingerprinting technique of automated ribosomal intergenic spacer analysis (ARISA). SWCNT exposure and time of exposure was found to significantly impact microbial community structure in relation to control environments void of SWCNTs, and this impact was differentially distributed, affecting some members of the microbial community more than others. Such impacts could significantly affect the often critical biological functions that microbial communities play in environmental maintenance and control.

Cytotoxic effects of SWCNTs during *E. coli* K12 biofilm development were investigated by Rodrigues and Elimelech (*11*). Bacterial biofilms are renowned for their resiliency and resistance to antimicrobials due to their production of a protective barrier of exopolymeric substances. Similar protective capabilities were shown against pristine SWCNTs. *E. coli* biofilms with exopolymeric barriers required 10 times higher concentrations of SWCNTs to reduce cellular biomass than those biofilms without exopolymer presence (200-400 μg SWCNTs/ml

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versus 20-40 μg SWCNTs/ml). Exopolymeric substances were similarly protective against MWCNTs in an activated sludge environment (*17*).

Although the direct contact effect of CNTs is likely the major contributor towards bacterial cytotoxicity, other factors can as well play a role. For example, several genetic systems related to oxidative stress, such as *soxRS* and *oxyR*, are expressed after exposure to SWCNTs and MWCNTs, which can instigate various forms of cellular damage associated with DNA, lipids, and/or proteins (*4*). The electronic structure of SWCNTs in terms of their metallic or semiconducting properties exhibits cytotoxic effects, with loss of *E. coli* K12 viability increasing with increasing fractions of metallic SWCNTs (*19*). Nanoparticles may also be taken up by cells and directly interact with DNA in vivo, thereby inhibiting bacterial growth (*20*). The presence of residual metal catalysts used in CNT synthesis, such as cobalt, that remain bioavailable to the bacterial cells may additionally affect growth rates (*12*).

3. C_{60} Nanoparticles

Due to their hydrophobic nature and poor water solubility, fullerene C_{60} nanoparticles were initially considered relatively benign towards biological systems. However, it was later discovered that when introduced into water, C_{60} forms aggregates (referred to as nC_{60} where n is the number of single C_{60} cages in an aggregate) that can exhibit cytotoxicity towards living organisms, including microorganisms. nC_{60} toxicity was typically attributed to oxidative stress effects and the generation of reactive oxygen species (ROS), but later studies have shown this not to be the case (*21*, *22*). However, the exact mechanism or mechanisms contributing to nC_{60} microbial toxicity (or non-toxicity) remains controversial and conflicting. Lyon and Alvarez (*23*) have proposed that nC_{60} requires direct contact with a bacterial cell whereupon it acts as an oxidant and disrupts membrane potential, resulting in loss of the proton gradient, interruption of cellular respiration, and cell death. Their studies demonstrated nC_{60} cytotoxic effects against *E. coli* K12 and *B. subtilis* within one hour at 5 μg/ml concentrations. They show in several additional studies that factors such as nC_{60} size (smaller = more toxic), exposure time and dose, and age of the nC_{60} preparation all impact cytotoxicity (*24*, *25*). Chae et al. (*26*) similarly demonstrated nC_{60} cytotoxicity against *E. coli* K12 and *Vibrio fischeri* over a 4 day exposure period at an nC_{60} concentration of 1.46 μg/ml. Kang et al. (*9*) demonstrated nC_{60} toxicity against *E. coli* and *P. aeruginosa* after impact onto nC_{60} coated filters, although minimal cytotoxicity was displayed against *B. subtilis*. nC_{60} cytotoxicity was also shown to be mitigated in the presence of NOM and soil particulates, possibly due to sorption disallowing direct nC_{60} cell contact and/or consequent changes to nC_{60} surface chemistry (*27*).

Using *E. coli* and *B. subtilis* as model Gram negative and Gram positive bacteria, respectively, Xia et al. (*28*) counter with their findings that nC_{60} is fully non-toxic against bacteria when prepared in the absence of tetrahydrofuran (THF) solvent, and it is the THF solvent or other preparative contaminants that contribute to cytotoxicity. Hadduck et al. (*29*) similarly showed a lack of

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nC_{60} cytotoxicity against *E. coli* and the yeast *Saccharomyces cerevisiae*. They discuss several factors that need to be considered when working with nC_{60} and microorganisms. Most importantly, THF even at trace amounts can generate derivatives toxic to microorganisms, and therefore must be removed from nC_{60} preparations or carefully controlled for. nC_{60} may also precipitate in certain types of microbiological growth media, resulting in lower than expected exposure doses. Additionally, organic components within the growth media may bind up nC_{60} thereby reducing its bioavailability. Under more real-world conditions using an activated sludge microbial community and the molecular fingerprinting method of denaturing gradient gel electrophoresis (DGGE), Nyberg et al. (*30*) demonstrated that nC_{60} exposure over a several month period had no effect on the microbial community structure. Indeed, when nC_{60} was added to an activated sludge biomass, nearly 88% of the nanoparticles were removed by supposed biosorption effects (*31*).

Discrepancies in nC_{60} cytotoxicity were recently acknowledged and addressed by Chae et al. (*5*) and somewhat attributed to differences in size, surface chemistry, and structural density of the nC_{60} particles linked to variances in preparation methods. Sonication of nC_{60} suspensions, for example, can increase their degree of hydroxylation, resulting in greater ROS generation. They showed that the smaller <50 nm fractions of C_{60} in nC_{60} aggregates are indeed more hydroxylated and more cytotoxic against *E. coli* K12, with even greater cytotoxicity demonstrated in the presence of ROS activating ultraviolet (UV) irradiation (a 4-log reduction in *E. coli* numbers when exposed to UV and <50 nm particles versus a 2.5-log reduction in *E. coli* numbers when exposed to UV and larger nC_{60} aggregates) (Figure 4). They additionally demonstrated cytotoxicity against MS2 bacteriophages. Thus, there exists evidence both for and against the cytotoxic effects of nC_{60} on microbial subjects, and more research is clearly needed to better validate and understand the interactions of nC_{60} within the microbial ecosystem.

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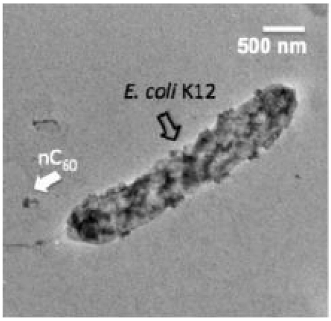

Figure 4. Electron micrograph of nC_{60} compromising the integrity of an E. coli bacterial cell membrane. (Used with permission from Chae et al. (5))

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4. Silver Nanoparticles

The antibacterial properties of silver (Ag) have been recognized for centuries, and it is for this reason that the current nanotechnological application of silver represents one of the most marketable consumer product lines available. Silver nanoparticles can be found, for example, in socks, bandages, cookware, toothbrushes, water filters, refrigerators, washing machines, paint and a host of other merchandising inventions targeted towards antimicrobial control, precisely because they are very good at what they do. Silver nanoparticles have been shown to portray broad spectrum microbicidal activity against a wide number of Gram negative and Gram positive bacteria, fungi, algae, and viruses/bacteriophages. For example, silver nanoparticles at 1-5 μg/ml concentrations were capable of fully inhibiting the growth of *P. aeruginosa* at 10^7 cfu/ml in liquid culture (*6*). *P. aeruginosa* biofilm formation was similarly controllable with silver nanoparticle addition at 1 μg/ml. Silver nanoparticles have even merged with nanotubes to produce, for example, silver-MWCNTs that have been shown to be more effective at killing *E. coli* than MWCNTs alone (*32*). The reader is directed to excellent recent reviews by Marambio-Jones and Hoek (*33*) and Fabrega et al. (*2*) for more examples.

Although the antimicrobial effectiveness of silver nanoparticles is well recognized, their mechanisms for promoting cytotoxicity are still not well understood, but can likely be attributed to several factors (*33*). Silver nanoparticles can release silver ions that may generate ROS, or silver ions may be taken up by the cell where they affect ATP production, DNA replication, and/or transport (Figure 5). Evidence that silver nanoparticles directly interact with cells and damage cell membranes also exists. The size, shape, solubility, and aggregation state of silver nanoparticles additionally affects antimicrobial efficacy. The conversion of normally hydrophobic silver nanoparticles to hydrophilic states has been shown to enhance bacterial cytotoxicity (*34*).

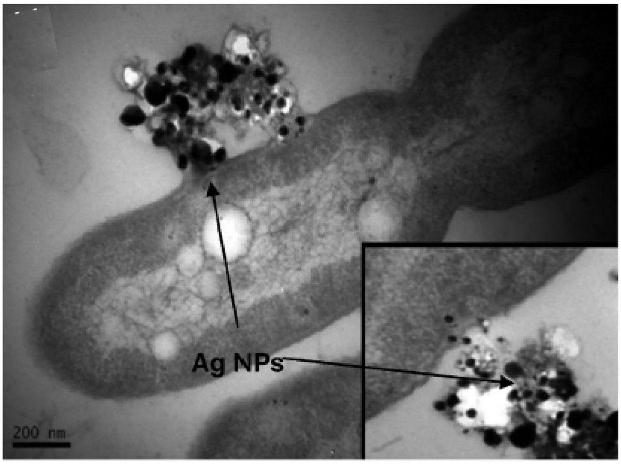

Figure 5. Silver (Ag) nanoparticles interacting with a Pseudomonas putida bacterial cell. Inset shows silver nanoparticle uptake by the cell. (Used with permission from Fabrega et al. (2))

Due to their exceptional antimicrobial properties and consequent widespread use, there is significant concern that the entry of silver-based nanoparticles into environmental ecosystems may be particularly disruptive. For example, Kim et al. (*35*) demonstrated the presence of silver-based nanoparticles in sewage treatment plants that heavily rely upon a population of 'good' bacteria to effectively carry out their wastewater treatment activities. The eradication of these beneficial bacterial populations would be clearly detrimental. Indeed, Liang et al. (*36*) do demonstrate that silver nanoparticles continuously loaded into a wastewater bioreactor system are capable of reducing beneficial nitrifying bacterial populations. However, Kim et al. (*35*) show that silver nanoparticles are transformed into possibly less toxic silver sulfide in the sewage sludge. Thus, the speciation of silver nanoparticles and the individual toxicity of each of these species must be considered rather than just the silver nanoparticles themselves when establishing toxicity profiles. The toxicity of silver nanoparticles is also reduced in the presence of common aquatic humic acids (*37*) and 97% of silver nanoparticles were shown to be removed from wastewater, likely through aggregation and sedimentation (*31*). Silver nanoparticles added to soil ecosystems within a concentration range from 3.2 to 320 µg/kg soil were capable of reducing microbial biomass in a dose dependent manner over 4 month exposure periods (*38*). In aquatic sediments, the addition of silver nanoparticles at up to 1000 µg/L concentrations showed minimal effect on bacterial community diversity as determined through DGGE profiling, presumably due to aggregation and other interfering physicochemical variables (*39*).

Although silver-containing nanoparticles are portrayed as an outcome of the nanotechnology revolution, it is more likely that nanoscale silver particles have been used commercially for the past 100 years, and it is only the 'nano' prefix that is new (*40*). Thus, ecosystems have likely been exposed to the cytotoxic effects of silver nanoparticles for decades and our newfound interest in understanding their environmental interactions may be overstated. However, their long-term use does not coincide with long-term study, and there exists comparatively little data on their environmental fate and activities in relation to microbial population effects. More research is needed, especially since silver nanoparticle toxicity translates to higher organisms as well, including zebrafish, clams, rodents, and humans (*40*).

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5. Metal Oxide Nanoparticles

Nanosized metal oxide particles can take various forms (cerium dioxide (CeO_2), chromium dioxide (CrO_2), molybdenum dioxide (MoO_3), zinc oxide (ZnO), bismuth trioxide (Bi_2O_3), indium tin oxide (InSnO), etc.), but the most prevalently applied metal oxide nanoparticles are titanium dioxide (TiO_2) and zinc oxide (ZnO). TiO_2 and ZnO nanoparticles are found, for example, in sunscreens, toothpaste, cosmetics, paints, and textiles. They both have been shown to exhibit excellent antibacterial activity against both Gram positive and Gram negative targets (Figure 6). Wei et al. (*41*), using TiO_2 at 100 to 1000 µg/ml concentrations and under photocatalytic (UV light) exposure, showed 100% mortality against *E. coli* within several minutes at 10^6 cells/ml in laboratory media. Jiang et al. (*1*), using ZnO at 20 µg/ml in the absence of photocatalytic exposure, demonstrated 100% mortality against *E. coli*, *B. subtilis*, and *Pseudomonas fluorescens* within 2 hours at 10^8 cells/ml in saline solutions. TiO_2 under similar non-photocatalytic conditions exhibited no significant toxicity, exemplifying its reliance on photocatalytic activation tied to ROS generation. However, even under dark conditions TiO_2 exhibits microbial toxicity by as yet unknown mechanisms (*42*). The cytotoxic mechanism of ZnO seems linked in part to its release of Zn^{2+} ions which were shown to affect bacterial growth rates for several microorganisms but then again not affecting others such as *E. coli* (*42*, *43*). Xie et al. (*44*), in their studies with *Campylobacter jejuni*, showed highly sensitive killing after exposure to 25-50 µg/ml ZnO concentrations with demonstrable upregulation of several stress response genes, indicating that oxidative stress also plays an important role. Other mechanisms attributed towards ZnO cytotoxicity include direct cell binding resulting in cell wall and cell membrane damage, intracellular accumulation of ZnO, and ZnO induced electrostatic forces that directly kill bacteria (*45*). Toxicity has been correlated to size, with smaller size metal oxide nanoparticles exhibiting greater cytotoxicity, presumably due to more efficient penetration into the cell or enhanced generation of radicals (*45–47*). Metal oxide nanoparticles have additionally been merged with other nanotechnologies, as exemplified by the encapsulation of TiO_2 nanoparticles into SWCNTs (*48*). Besides bacteria, TiO_2 and/or ZnO have also been shown to be effective antimicrobials against fungi, algae, and viruses/bacteriophages (*49–52*). Pelletier et al. (*53*) studied the effects of CeO_2 nanoparticles on Gram negative *E. coli* and *Shewanella oneidensis* and Gram positive *B. subtilis*. Bacterial toxicity was shown to be size dependent with smaller particles generally being more toxic. Interestingly, *S. oneidensis* exhibited no significant growth inhibition to CeO_2 nanoparticles, emphasizing the fact that nanoparticles in general can display heterogeneous effects across diverse microbial populations.

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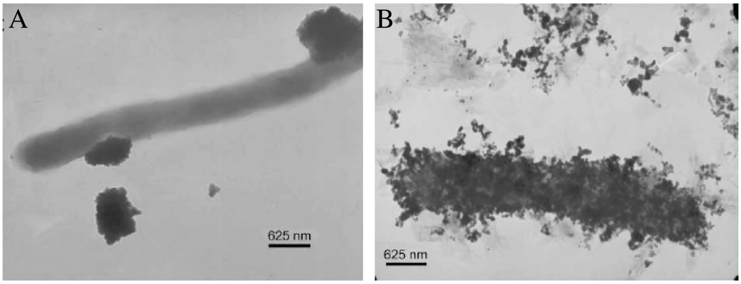

Figure 6. Electron micrographs depicting the attachment of (A) TiO_2 and (B) ZnO nanoparticles to a Pseudomonas fluorescens bacterial cell. (Used with permission from Jiang et al. (1))

The environmental impacts of metal oxide nanoparticles have been studied under real-world scenarios, although in limited format. The addition of ZnO nanoparticles at 10 and 50 µg/ml to a synthetic wastewater medium in a batch reactor resulted in an inhibition of biological nitrogen and phosphorous removal

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(*54*). Zn^{2+} ions were shown to affect the corresponding denitrifying bacteria but other critical members of the microbial activated sludge consortium, such as ammonia and nitrite oxidizing bacteria, were less affected. Water chemistry was shown to influence ZnO cytotoxicity, with *E. coli* surviving to a significantly greater degree in more complex media (i.e., ultrapure water versus Luria-Bertani media), presumably due to the generation of precipitates that removed Zn^{2+} from the medium thereby reducing toxicity (*43*). Limbach et al. (*55*) showed that up to 6% by weight of CeO_2 nanoparticles passed through to the effluent stream in a model wastewater system, with surface charge of the sludge flocs significantly influencing the removal efficiency of CeO_2 nanoparticles from the waste streams. Kiser et al. (*31*) demonstrated that only 23% of TiO_2 nanoparticles were removed from a wastewater biomass. Horst et al. (*56*) demonstrated that *P. aeruginosa* in aqueous media and natural waters were able to disperse TiO_2 nanoparticles, converting large agglomerates into smaller nanoparticles that associated with *P. aeruginosa* cells. In mixed microbial communities, this could increase TiO_2 nanoparticle toxicity among more sensitive microbial species. Using natural soil microbial community microcosms, Ge et al. (*57*) showed that the addition of TiO_2 or ZnO nanoparticles at 0.5 mg/g soil concentrations over 60 days were capable of affecting microbial community dynamics as measured through terminal restriction fragment length polymorphism (T-RFLP) DNA fingerprinting, with ZnO nanoparticles showing greater community disruption than TiO_2 nanoparticles. Kim et al. (*7*) added ZnO to soil microcosms at 2 mg/g soil and demonstrated over an 8 week period a decrease in enzyme activity associated with microbial nutrient cycling.

6. Quantum Dots

Quantum dots are approximate 2-10 nm diameter semiconductor nanocrystals consisting of a core typically comprised of cadmium-selenide (CdSe), cadmium-telluride (CdTe), zinc-selenide (ZnSe), or lead-selenide (PbSe) surrounded by a zinc or cadmium sulfide shell. They possess interesting optical properties such as high quantum yield and photostability that enables their use in cell labeling and biomedical imaging technologies as well as in various electronic and solar cell applications.

Quantum dots do exhibit microbial cytotoxicity but with dependence on surface chemistry, stability of the core and shell materials, and presence/absence of light. For example, when exposing *P. aeruginosa* cultures to stable, non-functionalized CdSe quantum dots, no toxic interactions were observed at up to 675 nM concentrations over a 24 hour period (*58*). Mahendra et al. (*59*) showed similar minimal interactions of pristine CdSe quantum dots with *P. aeruginosa*, *E. coli*, and *B. subtilis* at 20 nM and lower concentrations over 48 hour exposure periods. However, destabilization of CdSe quantum dots after exposure to high and low pH significantly increased cytotoxicity (near 100% mortality) due to rapid release of known bactericidal cadmium and selenite ions. CdSe quantum dots have similarly been shown to destabilize under environmentally relevant oxidative conditions (*60*). Interestingly, Mahendra et al. (*59*) observed that destabilized CdSe quantum dots were more toxic to the bacteria than equivalent concentrations of the cadmium and selenite salts alone, inferring that other factors additionally contributed towards cytotoxicity. Priester et al. (*3*), in direct comparisons between CdSe quantum dots and free cadmium ions, showed that *P. aeruginosa* cells also exhibited membrane damage, intracellular accumulation of quantum dots, and both extracellular and intracellular ROS generation (Figure 7). Zhang et al. (*61*) additionally described the uptake of CdSe quantum dots by *E. coli* and consequent direct binding to DNA, which can potentially result in nicking, aggregation, and other genotoxic effects that contribute to cell death. The ability of bacteria to take up quantum dots is increased in the presence of light, presumably due to resulting oxidative damage to the cell membrane permitting such entry (*62*).

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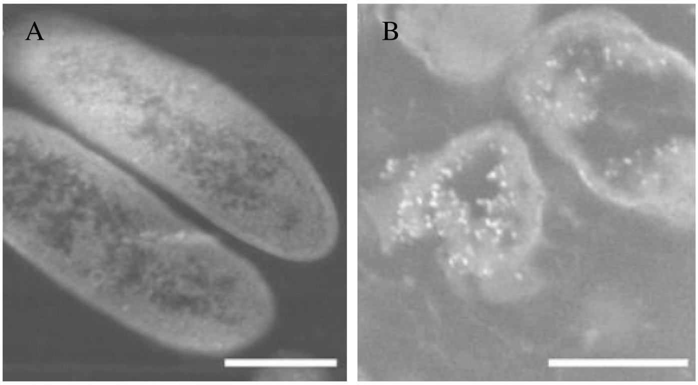

Figure 7. Electron micrographs of Pseudomonas aeruginosa bacterial cells grown in the (A) absence of quantum dots and in (B) the presence of CdSe quantum dots where cell membrane damage becomes evident. Bar = 1 µm. (Used with permission from Priester et al. (3))

CdTe quantum dots are similarly cytotoxic, demonstrating differential toxicity against *P. aeruginosa*, *E. coli*, *S. aureus*, and *B. subtilis* at 200-400 nM concentrations over 3 to 10 hour exposure periods with ROS generation contributing significantly to mortality (*63*). Algae exposed to CdTe quantum dots at as low as 0.1 µg/ml concentrations were shown to respond via upregulation of several stress response genes (*64*). The release of cadmium ions is also cytotoxic, and it has been shown that bacteria themselves can degrade and destabilize CdTe quantum dots sufficiently to promote the liberation of cadmium ions (*65*).

P. aeruginosa, under both CdSe and CdTe quantum dot exposures, exhibits lower mortality presumably due to the protective effects of its exopolymeric layer preventing direct cell/quantum dot binding and consequent direct oxidation. Dumas et al. (*66*) looked at direct binding more closely in their work with CdTe quantum dots and *E. coli*, *P. aeruginosa*, *S. aureus*, and *B. subtilis*. Whereas CdTe quantum dots were shown to directly bind to all bacteria, binding was more prevalent in Gram positive bacteria (*S. aureus* and *B. subtilis*) than in Gram negative bacteria (*E. coli* and *P. aeruginosa*), with significant membrane depolarization occurring in Gram positive bacteria but not in Gram negative.

However, Gram negative bacterial mortality was significantly greater than Gram positive. Indications were that the generation of hydroxyl radicals was the most important factor in determining cytotoxicity, with direct cell binding and exposure to free Cd^{2+} playing less significant roles.

In terms of real-world interactions of quantum dots with environmental microbiota, relatively few studies have been performed. Gao et al. (*67*) looked at CdSe quantum dot toxicity in freshwater sediment slurries. CdSe quantum dots added at concentrations up to 0.2 μg/ml did not significantly affect the microbial population as measured by nitrate reduction. Humic acids and NOM at environmentally relevant concentrations tended to show little effect on CdSe quantum dot stability or aggregation potential (*68*, *69*). In an interesting environmental food web experiment, Werlin et al. (*70*) demonstrated that CdSe quantum dots internalized by *P. aeruginosa* bacteria could be transferred largely intact to protozoa (*Tetrahymena thermophila*) grazing on the *P. aeruginosa* bacterial population. Furthermore, since protozoa ingest not one but rather many bacterial prey, there is a biomagnified accumulation of CdSe quantum dots within the protozoa, which then remains potentially bioavailable to the next higher trophic level organism in the food chain.

7. Environmental Implications

Predicting the impact of manufactured nanomaterials on microbial communities and translating those impacts into broader environmental consequences is a formidable task, and our current understanding of nanotoxicology as it applies to the microbial world is simply too inadequate at this time to formulate robust models for doing so. Although nanomaterial cytotoxicity is apparent among microbial species, the minimalism of most experiments performed to date do not translate well to the complex real-world environmental ecosystem. Single species microbial cultures or nominal mixed cultures growing within defined laboratory media, for example, are not representative of the complex free or biofilm associated microbial consortia coexisting within equally complex soil, water, and wastewater environmental matrices. Measuring ecotoxicity in the absence of distinctly influential variables such as NOM and solution ionic strength is clearly not representative of natural conditions, which, in conjunction with the likely over-addition of nanoparticles over short exposure periods, drives experimental results towards positive toxic outcomes. However, data collected in these modest yet still pioneering research efforts are essential for forming the knowledge database required for progress towards more realistic microorganism/ecosystem/nanomaterial interactions. And indeed, microbial community dynamics in soil, sediment, freshwater, and wastewater environments impacted by simulated nanomaterial influx are beginning to be assessed through various gene-based community profiling methods (*18*, *30*, *39*, *57*). However, these experimental models are still in very early stages and definitive conclusions on what nanomaterial impacts may trigger within natural microbial populations remains obscure.

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Considering that early evidence predicts at least some level of nanomaterial toxicity towards microorganisms, we can likely assume that ecosystem microbiota will undergo certain transformations upon exposure that may or may not be significant. Taking the more vigilant route and correlating an increase in nanomaterial production with increased unintended environmental exposures, we can at least contemplate potential deleterious consequences, the most significant of which would be a loss of microbial community structure resulting in altered biogeochemical processes, including nutrient cycling and maintenance of food webs. Considering the key roles that microorganisms play in carbon, nitrogen, sulfur, phosphorous, and other mineral cycling, loss of activity and balance at this fundamental level would be significantly detrimental. However, bacterial populations are known to be sufficiently robust that recovery of population balance may develop fairly quickly. Less envisioned are the evolutionary consequences of microbial gene exchange mechanisms. Release of chromosomal and extra-chromosomal DNA upon nanomaterial-induced cell lysis can influence rates of horizontal gene exchange carried out by transformation (the uptake of DNA from the environment by a competent recipient bacterium), although the ability to observe increased rates of transformation above low naturally occurring rates using currently available tool sets would be difficult (*71*). Transduction, the transfer of DNA from one cell to another by bacteriophages, may be more detrimental, however. As bacteriophages prey on bacteria, they routinely pick up and transfer genetic material between and among their bacterial hosts, and with such efficiency and tenacity that they disseminate enormous influences on microbial diversity, genetic exchange, and bacterial population dynamics within the environmental microbiota. In aquatic environments alone it is estimated that transduction occurs at the phenomenal rate of 2×10^{16} times per second (*72*). As a result, 10 to 20% of a bacterial genome can often be traced to a phage origin, thus endowing bacteria with traits such as bacteriocin activity, pathogenicity, and additional nucleic acid integration/excision mechanisms (*73*). The cytotoxicity of nanomaterials towards both bacteria and bacteriophages would seem influential towards affecting the evolutionary pace of transduction events.

The synthesis of effective risk-based models for describing nanomaterial cytotoxicity is additionally hampered by a corresponding lack of interrelated parameter data (*74*). Little is surprisingly known, for example, on production volumes of nanomaterials, and thus attempting to predict quantities potentially released into the environment is at present futile (*75*). And once introduced within an ecosystem, knowledge pertaining to nanomaterial fate, transport, persistence, bioaccumulation, and biomagnification is wholly lacking. Nanotoxicology in general begs for more realistic experiments that take into account microbial diversity and ecosystem complexity on a larger scale, with infusion of a tool box of ecological, ecotoxicological, microbiological, chemical, engineering, statistical, and risk assessment methods and practices.

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Chapter 7

Methodologies for Toxicity Monitoring and Nanotechnology Risk Assessment

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While many engineered nanomaterials are commonly used in commercial products, interactions with biological systems, transport, kinetic, toxicity and accumulation in living organisms as well as their environmental and health effects are largely unknown and their use has recently become of particular concern. Due to their specific physical and chemical properties and increased reactivity, nanoparticles may interact with biological tissues and cause toxicity. Understanding the fundamental mechanisms by which nanoparticles induce toxicity and assessing cytotoxic response is of particular interest. Parameters like surface charge, surface coating, surface area, particle reactivity, composition, aggregation and dissolution may all affect cellular uptake, *in vivo* reactivity and distribution across tissues. This chapter describes methodologies for nanotechnology risk assessment and provides an overview of recent efforts to develop predictive models of nanoparticle induced toxicity. Interaction of nanomaterials with proteins, cells and tissues and their cytotoxic response in model biological systems in relation to the physico-chemical properties are discussed. Special emphasis is given to the use of zebrafish embryos as a model toxicological target and as a screening tool for toxicity risk assessment. Biophysical

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characterization methods at the nano/bio interface and the use of sensors as predictive tools for toxicity monitoring are discussed in detail.

1. Introduction

Nanoparticles and nanostructures are materials engineered at the nanometer scale (dimensions of the order of or below 100 nm) (*1–4*). Rapid progress of nanotechnology and advanced nanomaterials production over the past decade offer significant opportunities for a wide range of applications in medical diagnostics, imaging and drug delivery to sensing, catalysis and environmental remediation (*5*, *6*) (*7–10*). Significant advancement has been made in the control of chemical composition, size, size distribution and shape of nanoparticles (*11*, *12*). The small size creates new properties different from the bulk materials. The uniqueness of these materials is due to their mechanical, electrical, optical, catalytic, magnetic and photonic properties, and extremely high surface area. These features make them attractive in many fields ranging from biomedical applications to electronics and energy production. Various programs to promote applications of nanotechnology have been initiated worldwide. At the same time, there is concern that these properties could also have negative impact on human health and the environment (*5*, *13*, *14*). Nanoparticles may have enhanced reactivity (*15*) due to their nanometer size (*16*) that cannot be predicted by the bulk properties of the corresponding macroscopic size material. Their properties largely depend on size, charge and surface coverage and, in biological media, on the entry sites of the biological entity encountered during exposure (*17*) (*18*).

While many nanomaterials are commonly used in commercial products, interactions with biological systems, transport, kinetic, toxicity and accumulation in living organisms as well as their environmental and health effects are largely unknown and their use has recently become of particular concern (*19*, *20*). Nanoparticles can penetrate biological systems through various routes. Whether the particles cross cellular membranes, interact with cells, organs and tissues, accumulate at different locations in the body, stay inert or interfere with normal physiological process, will determine their behavior, fate and transport in biological systems (*21–23*). Since cells and tissues are in direct contact with the nanoparticle's surface, these effects are strongly related with the material's surface chemistry (*24–32*). Thus, parameters like surface charge, surface coating, surface area, particle reactivity, composition, aggregation and dissolution may all affect cellular uptake, in vivo reactivity and distribution across tissues (*6*, *30*, *33–35*). These parameters are important because cells/tissue will first "see" the surface of the nanoparticle, which will dictate their toxicological response. In addition, nanoparticles can gain access to cellular and tissue locations that are inaccessible to larger particles (*5*, *36*).

Due to these potentially harmful effects, concerted efforts to conduct fundamental, interdisciplinary research for understanding the behavior of nanomaterials in biological systems and study their possible interactions with living organisms have been promoted at a global scale (*37*). Ongoing research efforts focus on assessing safety risks of nanotechnology and define "*nanomaterials – induced toxicity"*. Most materials studied to date are carbon nanotubes, fullerenes, gold and silver nanoparticles. Most studies have been performed using conventional cell culture methodologies and viability assays. There are relatively few data on the effect of surface coating, size, surface coverage, charge and shape. Recent research highlights (*27*, *38–40*) have stressed the need to develop a "predictive toxicological paradigm" for the assessment of nanomaterial toxicity in which the material's physicochemical properties that "leads to molecular or cellular injury and also has to be valid in terms of disease pathogenesis in whole organisms" has to be considered (*27*). The challenge is to identify key factors to predict toxicity, permit targeted screening, and allow controlled generation of new, safer nanoparticles based on structure-toxicity information. Research in this direction is expected to enable development of new methods and models for predicting the potential impacts of the next generation of nanoparticles and nanostructures on health and the environment.

This chapter describes methodologies for nanotechnology risk assessment and recent efforts to develop predictive models of nanoparticle induced toxicity. In the first part of this chapter, interaction of nanomaterials with proteins, cells and tissues and their cytotoxic response in model biological systems in relation to the physico-chemical properties are discussed. In the second part, biophysical characterization methods at the nano/bio interface and the use of sensors as predictive tools for toxicity monitoring are discussed in detail. Special emphasis is given to the use of zebrafish embryos as a model toxicological target and a screening tool for toxicity risk assessment.

2. Nanoparticles As Emerging Contaminants. The Effect of Physico-Chemical Parameters

Nanoparticles can have hazardous effects due to their small sizes, enhanced surface area and increased reactivity. Depending on the type and nature of particles, their chemical reactivity causes an increased production of reactive oxygen species (ROS). Several metal oxide and metal nanoparticles and carbon nanotubes have been shown to induce free radical production. Thus, oxidative stress, inflammation, DNA, mitochondrial and tissue damage might occur (*41*). The presence of nanoparticles in a cell culture has been shown to affect immune response, resulting in an increased production of inflammatory cytokines (*42*). Due to their small size, nanoparticles can interfere with biological processes, cross biological membranes, access cells, tissues and organs, accumulate and move from one location to another in the body. The interaction of these particles with the biological environment, proteins, cells and tissues is a function of their surface reactivity, composition, size and shape. Modifications of nanoparticles surface coating with biocompatible materials can potentially result in reduction or absence of toxic effects, including cell death and inflammation.

Recent reports have demonstrated that various types of nanostructures (e.g. carbon nanotubes, Pt, Ag, Au, ZnO, CuO, TiO_2) show varying degrees of cytotoxic effects which are not observed with larger particles or the bulk material

(*43*, *44*). These focus on assessment of health effects of nanoparticles after respiratory, gastrointestinal and dermal exposure using mice (*15*, *45*), cell culture (*43*, *46*, *47*), bacteria, crustaceans (*48*), freshwater alga (*44*) and zebrafish (*49–65*). While research is beginning to determine mechanisms of nanoparticle toxicity, this subject remains largely unexplored (*50*). Understanding the translocation, accumulation and retention pathways in vital target sites needs more systematic studies (*4*). The mechanisms of nanoparticles' toxicity are complex (*66*) and may be a result of:

- *nanomaterial size*: All nanoparticles within the 2–100 nm size range were found to alter signaling processes essential for basic cell functions. Recent research suggests that nanoparticles less than 100 nm in diameter can enter cells, those with diameters below 40 nm can enter the cell nucleus and those that are smaller than 35 nm can pass through the blood–brain barrier and enter the brain (*45*, *67–71*). Nanoparticles can be both potential carriers of signaling molecules as well as individual entities capable of interacting with cellular structures in their relevant dimensional range and sometimes cause contradictory bioeffects.
- *material solubility* which varies with particle composition and species (*44*, *66*, *72*). Nanoparticles with very low solubility can be persistent within the biological system and induce long-term effects on the organism. Moreover, nanoparticles with high solubility can release soluble ions, which can be toxic.
- *aggregation and surface charge* are other important factors that determine cellular uptake and nanoparticle adsorption. Aggregation results in entities ranging from several hundred nm to several µm (*44*). Nanoparticles placed in an aqueous environment might quickly aggregate due to electrostatic interactions. Formation of large size aggregates can prevent cellular uptake and bioaccumulation. Scanning electron microscopy (SEM), transmission electron microscopy (TEM) and particle size distribution (PSD) analysis can be used to determine the status of nanoparticles aggregation. Fluorescently labeled nanoparticles can be used to facilitate assessment of nanoparticle accumulation, and degree of internalization from target membranes.
- *production of oxidant species*: nanoparticles in contact with biological materials (cells or tissues) can trigger production of ROS (*73–75*) that can further damage the cells through oxidative stress (*43*, *44*, *48*).
- *reactivity* due to the metabolic alkalosis or intracellular dissolution (*15*, *66*, *75*)
- *increased mobility across cell membranes*: nanoparticles may be able to cross cell membranes (*76*). However, studies have shown that nanoparticles do not necessarily have to enter the cells to produce toxic effects (*48*).
- *surface coating:* coating and physicochemical properties (hydrophobicity and surface charge) (*77*, *78*).

Several studies have shown a direct relationship between nanoparticle structure and their impact on biological systems (*65*, *79*). For example, gold nanoparticles - traditionally an inert material as a bulk compound - show different biological responses in mammalian cells. In some studies, Au nanoparticles have shown reduced toxic effects despite their uptake into the cells by endocytosis (*80*, *81*). However, when the particles were functionalized with cationic side chains they were toxic (*82*). The aggregation state and surface charge are other important factors that determine cellular uptake and nanoparticle's adsorption on cellular membrane. The surface coatings for the 'same' type of material may depend on synthesis and/or processing used (*83*, *84*). Research efforts in this field suggest the need to correlate the material's biophysical characteristics with *in vivo* and *in vitro* assays and establish a "life cycle" of the behavior and transport of nanoparticles in biological systems starting from the material processing to cellular uptake, tissue response and clearance.

Toxicity of nanostructures varies according to tissue/cell types and *in vitro*/*in vivo* model used. Generally, cytotoxic effects emerge in a dose- and time-dependent manner for all types of particles. Particle aggregation and particle dissolution has also been suggested as a factor in the cytotoxicity of nanoparticles. The toxicological effects of carbon-based materials, such as fullerenes and single-walled nanotubes have been the most extensively investigated. Comparison studies reported that single wall carbon nanotubes have greater toxicity than other carbon nanoparticles (*85–87*). The most widely used nanoparticles are metal (e.g. Au, Ag and Pt) and several types of metal oxides like titania, iron oxides and zirconia. The most widely used nanoparticles and a summary of their toxicological effects are provided below:

Metal nanoparticles. Gold (Au) nanoparticles have been extensively used due to their many interesting optical and catalytic properties. Au nanoparticles are used as catalyst in many industrial (*88*, *89*) and biomedical applications, for example for targeting, imaging and therapy of cancer cells (*90*, *91*). Au nanoparticles are able to bind strongly to proteins containing thiol and amine groups (*92*, *93*). Li et al have studied the effect of Au nanoparticles on MRC-5 lung fibroblasts and found increased levels of hydroperoxide radicals which could react with proteins and induce DNA damage (*94*). In another study, Au nanoparticles with small diameters (~5 nm) caused lipid peroxidation. Oxidative stress and cytotoxicity induced by small Au nanoparticles were higher than those induced by particles with larger diameters (*95*). Another metal, silver (Ag) is well-known for its antibacterial properties. Silver nanoparticles are commonly used as antibacterial coatings of many biomedical products such as wound dressings and silver impregnated catheters (*96*). *In vitro* toxicity studies showed that the toxicity of Ag nanoparticles is concentration dependent (*97–99*). *In vivo* studies on zebrafish embryos demonstrated that small size Ag nanoparticles can diffuse into the embryo through the chorion pore channels; the number of dead embryos increased with the concentration of the nanoparticles (*98*). In another study on zebrafish, high concentrations of Ag nanoparticles (50 μg/mL) induced developmental malformations, low heart rate and degeneration of body parts (*99*). A recent study on four different shapes of platinum nanoparticles (nanoflowers, spheres derived from flower-shape, multipods, spheres derived from multipods) has concluded that these nanostructures do not show significant cytotoxic response and that they do not induce oxidative stress (*100*). In another study on zebrafish, the toxicity of different shape and size nickel nanoparticles has been investigated (*1*). 30, 60 and 100 nm Ni nanoparticles were less toxic than larger dendritic aggregated structures. Chen et al. have conducted a comparative, *in vivo* toxicology study of copper nanoparticles, micrometer size Cu particles and Cu ions on mice. Nanoparticles and soluble ions were found as moderately toxic while microsize Cu showed no cytotoxic effects (*101*).

Metal oxide nanoparticles. Metal oxides are extensively used in personal care products, textiles, environmental remediation (*102*) and biosensing. Several cytotoxic studies have been reported recently (*103–108*). When comparing the toxicity of nano and micrometer particles of several metal oxides (Fe_2O_3, Fe_3O_4, TiO_2 and CuO) in A549 human cell lines, it was found that CuO nanoparticles were more toxic than micrometer size CuO particles, causing significant mitochondrial damage (*103*). In the same study, TiO_2 caused more DNA damage. Studies have shown that TiO_2 can also induce ROS production. These nanoparticles can penetrate the epidermis and may have carcinogenic and inflammatory effects (*109*). TiO_2 nanoparticles can be dangerous for health via inhalation. Mice exposed to 2-5 nm particles through inhalation caused moderate inflammatory response (*110*). Iron oxides nanoparticles showed low toxicity and no differences among different size particles. Toxicity of TiO_2, Al_2O_3 and carbon nanotubes on bacterial strains was dependent on their chemical composition, size, surface charge and shape, but also on the bacterial strain (*204*). Recent studies with ZnO and CuO nanoparticles correlated toxicity with their dissolution in aqueous medium, producing soluble Zn^{2+} and Cu^{2+} ions (*111–113*). Solubility strongly influenced the cytotoxic effect (*107*).

3. Predictive Models for Assessing Toxicity

In order to determine whether a substance results in toxicity to humans, testing on biological tissue is required. Testing has involved a number of models from whole animals to use of cell culture. Combination of information from each of these models provides critical information for the evaluation of whether the substance will be toxic and also determines what level of exposure is safe to the general population. Toxicological studies are also important to assess health risks and deleterious side effects. A recent study has shown a direct relationship between the structure of nanoparticles and their toxicological impact (*65*, *79*). Changes in the size, chemical composition, surface structure, solubility, and shape may influence the biological effects of nanoparticles since these factors can alter protein binding and cellular uptake. Nanoparticle binding to proteins may generate complexes that are more mobile and can enter tissue sites that are normally inaccessible by free particles. Methodologies for toxicity screening include cell culture and *in vivo* systems, e.g. whole organisms and embryos. *In vivo* fate of nanoparticles, e.g. stability and bioaccumulation, and subsequent bioeffects, depend on a plethora of factors ranging from metrology of exposure (*114*), entry point, coating/capping, including the “adsorbed protein corona”

(*115*), biocompatibility and physico-chemical properties (*116*). The first step after nanoparticle's exposure in a biological environment is the rapid adsorption of proteins. The type, amount, and conformation (*117*, *118*) of the adsorbed proteins regulate cellular adherence, cell migration, proliferation, and differentiation (*119*, *120*).

The advantage of using cellular assays is that specific biological and mechanistic pathways can be isolated and investigated under controlled conditions, which is not feasible with *in vivo* techniques. In addition, spectroscopic and electro-optical methods can be used on cellular cultures to analyze fundamental processes involving nanoparticles at single cell level (e.g. crossing of cell membrane). Interaction of nanoparticles with cells is determined by factors like: electrostatic and hydrophobic interactions, specific chemical interactions as well as particle size (*121*, *122*). The uptake of nanoparticles by cells consists of attachment of nanoparticles to the cell membrane followed by internalization. Nanoparticles can alter membranes, change gene expression, disrupt mitochondrial function, form ROS, and decrease cell viability. Yet, the mechanisms by which extra-cellular compounds are recognized by and/or gain entry into target cells are largely unclear (*123*). For example, the toxic effect of copper ions and nanoparticles (*124*, *125*) was associated with mitochondrial failure, ketogenesis, fatty acid beta-oxidation, and glycolysis, while that of TiO_2 nanoparticles was attributed to changes in cell surface, membrane breakage and oxidative stress (*126*). Moderate levels of intracellular iron oxide nanoparticles affects neural cell functioning (*36*). CdSe quantum dots elevate cytoplasmic calcium levels in primary cultures of rat hippocampal neurons (*127*). The attachment of the particles to the cell membrane seems to be most affected by their surface charge, whereas cellular uptake may occur by pinocytosis, non-specific or receptor-mediated endocytosis or phagocytosis. Patil *et al.* showed that cells accumulate nanoparticles to a greater extent when the surface charges on the particles are negative demonstrating that electrostatic interactions are important determinants of protein adsorption and cellular uptake (*128*). Cell membranes possess large negatively charged domains, which should repel negatively charged nanoparticles. However, Wilhelm et al. (*129*) suggested that the negatively charged particles bind at cationic sites that form clusters on the cell surface because of their repulsive interactions with the negatively charged domains of the cell surface. In addition, the nanoparticles, already bound on the cell surface, present a reduced charge density that may favor adsorption of other free particles. Thus, the high cellular uptake of negatively charged nanoparticles is related first to the non-specific adsorption on the cell membrane and second to the formation of nanoparticle clusters (*128*).

The use of *in vivo* systems, on the other hand, provides information on the way the nanoparticles interact with target organs (e.g. intestine, digestive system, muscles, cartilages) as well as tissue response. A recent study has tried to establish whether *in vitro* assays can be used to predict lung damage following inhalational exposure of ZnO nanoparticles *in vivo* in rats. *In vivo* studies showed short-term lung inflammatory or cytotoxic responses while *in vitro* cell culture exposure produced minor responses only at high doses (*105*). This study suggests that cell culture methods might not accurately predict the *in vivo* exposure response (*130*).

Nonetheless, *in vitro* assays may provide a simple, readily available cytotoxic test (*107*) but the value of such testing is not fully established. In future research, *in vitro* cellular systems will need to be developed, standardized, and validated (relative to *in vivo* effects) in order to provide useful screening data on the relative toxicity of nanoparticles (*131*). To understand the mechanisms for nanoparticle toxicity, further refinements of cellular platforms are required in order to better mimic the *in vivo* conditions. Use of both *in vitro* and *in vivo* systems could allow for determination of the predictive ability of nanoparticles by cell culture studies on whole organism exposure. In the following sections we discuss methodologies for evaluating the toxicity of nanoparticles using both *in vitro* and *in vivo* systems.

4. Risk Assessment Using Sensors Technology on Cellular Platforms

Cell fate and development depends on a plethora of signals coming from the cell environment (*132*). The cell can be seen as a living transducing and amplification system that is capable of responding to environmental stressors (e.g. nanoparticle exposure) and provide relevant toxicity information. Changes in membrane fluidity, cell proliferation, cell morphology, electric parameters, cell-cell communication and adherence can all be used as sensitive indicators of cell- nanoparticles interaction. In conjunction with optical assessment and specific cell viability assays, one can derive dose-response curves that would hopefully extend the relevant concentration range towards a lower, more physiological one. Response curves depending on type of material, size and surface coatings will provide a better understanding of the physicochemical basis of nanoparticles-biological cell interaction, as a possible tool in rational design of novel nanostructured materials. Aiming for a better understanding of the intricate effects of nanoparticles at cellular level, the assessment of “gentle”, nonlethal effects on various cellular structures and processes is also possible using biophysical assays.

4.1. Potential Cellular Targets and Cell Mechanisms

The propensity of nanoparticles to cross cell barriers, enter cells and interact with subcellular structures is well established. Nanoparticles below 100 nm can enter the cell by caveolae, clathrin mediated endocytosis, lipid rafts, or diffusion. This can lead to substantial accumulation within mitochondria and the cell nucleus (*133*) triggering concerns regarding impaired oxidative balance and potential genotoxic effects. Nanoparticles can alter membranes, change gene expression, disrupt mitochondrial function, form ROS, and decrease cell viability (Figure 1). The type, amount, and conformation (*134*, *135*) of adsorbed proteins on nanoparticles regulate cellular adherence, cell migration, proliferation, and differentiation (*136*, *137*). Nanoparticles may generate ROS directly (*138–140*) or indirectly (by altering the function of mitochondria or NADPH oxidase) (*141–144*).

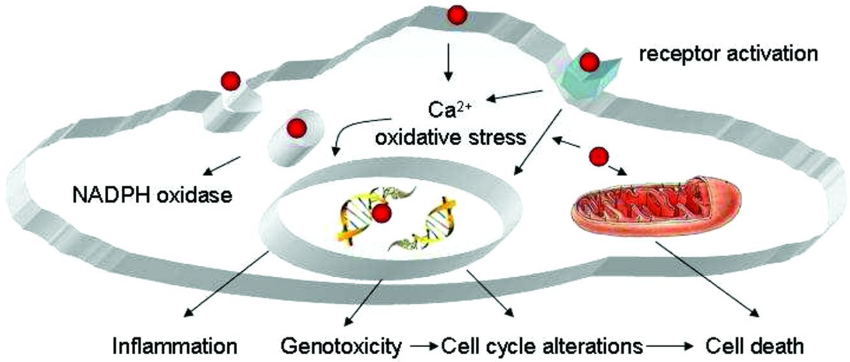

Figure 1. Potential cellular targets and cell mechanisms implicated in cell-nanoparticle interaction.

The presence of nanoparticles in a biological environment can cause inflammation, oxidative stress, activation of signaling pathways, genotoxicity and carcinogenicity. At the cellular level, the effects are possibly mediated by stress responses, DNA damage, carcinogenesis, mutagenesis, cell cycle perturbations, cell death, changes in cell differentiation and extracellular matrix contacts, and inflammation. For example, a toxic effect, in the case of TiO_2 nanoparticles, was attributed to changes in cell surface, membrane breakage and oxidative stress (*145*), while elevated cytoplasmic calcium levels were identified in primary cultures of rat hippocampal neurons challenged with CdSe quantum dots (*146*).

4.2. Microfluidic Platforms

Microfluidic biochips or lab-on-a-chip systems are modern technologies for biological analysis as they allow spatial and temporal control of growth conditions (ideally as close as possible with *in vivo* ones), application of small perturbations to the cellular microenvironment and monitoring the kinetics of the resulting cellular response. Microfluidic devices are alternative platforms to the conventional cell cultures and the well validated toxicologcal evaluations (e.g. MTT - 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (*147*), lactate dehydrogenase - LDH, calcein, caspases, DNA oligonucleosomal fragmentation as well as inflammatory cytokines) where the quantification of cell death is considered relevant for the onset of toxic insult. In contrast, dynamic evaluation of cellular platforms on an integrated biochip enables detection of subtle/nonlethal effects of nanoparticles and has the potential to provide better predictive information and a deeper mechanistic understanding. In a recent example (*148*), silver nanoparticles were able to induce abnormal cellular morphology, displaying cellular shrinkage and acquisition of an irregular shape, micronucleus formation and significant induction of genes associated with cell cycle progression as supported by the DNA microarray analysis, even at levels below cytotoxic doses. Likewise, inhibition of differentiation of stem cells (*149*) occurs below cytotoxic concentrations of silica nanoparticles, suggesting that nanoparticles risk assessment requires new, more refined approaches.

Microfabricated biochips are developed to continuously monitor cell behavior in a non-invasive manner (for a recent review see Wu et al. (*150*)), while some commercial optical devices can be found for instance at IBIDI (www.ibidi.de), Cellasic (www.cellasic.com), Dolomite (www.dolomite-microfluidics.com), and combined optical and electric assays at Acea Biosciences (www.aceabio.com)). They have demonstrated the ability to provide, in real-time, quantitative, high sensitivity data, paving the way for standardized cellular platforms (with controlled cell position, cell shape, cell polarity and internal cell organization) for *in vitro* assessment of nano-toxicity. Cellular dynamics are monitored via imaging or fluidic integration with microanalytical devices to detect metabolites or proteins produced by the cells.

4.3. Biophysical Methods To Characterize the Interaction of Nanoparticles with Cells

While optical evaluations (fluorescence and/or contrast methods i.e. phase or differential interference) are the preferred option, other electro-optical and scanning probe techniques have been recently used as powerful methods for characterization of cells in relation to external stimuli (e.g. particles). Alternative methodologies include: Surface Plasmon Resonance (SPR), Total Internal Fluorescence Microscopy (TIRFM) and Atomic Force Microscopy (AFM). These techniques allow detailed investigation of the bio-interfaces and processes at the cell membrane, as well as dynamic assessment (*151*, *152*) of cell growth, morphology and progression of the cell cycle. Thus, an ideal "real-time cellular platform" (*153*) includes multi-probe imaging with AFM, optical and SECM (scanning electrochemical microscopy) modes, which provides topological information and biochemical reactions at the local area of the interior and exterior of a cell while cell interaction with the extracellular matrix could be based on SPR, TIRFM and electric approaches using a nano-fabricated substrate. More recently, electrochemical microsensors have been used in several configurations to assess functional changes in nanoparticles-exposed immune cells and evaluate cell- nanoparticle interactions (*154*).

4.3.1. Impedance Spectroscopy To Study Cells

Electrical Impedance/dielectric Spectroscopy (EIS) is a modern method to study cellular systems based on the different frequency signatures of the conductive and capacitive elements within the particular structure of living cells and cell–electrode interaction. EIS provides real-time kinetics, live cell quality control (*155*, *156*), cell mobility, adherence and morphology (*158*, *159*), high information content (*160*), including cell-cell communication (*152*) and good sensitivity and reproducibility in monitoring an entire cell population. While fully automatable and label free, the inherent sensitivity and ability to eliminate stray effects are dependent on the electrode geometries, measurement set-up, integration of controlled flow through capabilities and complementary analytic methods (e.g. optical). Since the use of EIS to assess cytotoxicity has been demonstrated (*161*, *162*) in an electric cell-substrate impedance sensing approach (*163*), constant efforts are directed to development of optimized electrode geometries and measurement concepts (e.g. differential setups (*164*) and interdigitated electrodes IDEs (*165*, *166*)). Planar electrodes were optimized to examine *in vitro* or *in vivo* cell functions such as cellular metabolism (*167*), attachment and spreading of epithelial MDCK cells (*160*, *168*), and cell response to different toxic materials. On-line and continuous system based on ECIS for the monitoring of cell growth and cytotoxic effects of metal compounds have been developed (*155–157*, *169–176*). Besides enabling the assessment of target extracellular compound effects on adherent cell types, these cellular platforms are ideally positioned for more general cytotoxic evaluation of complex mixtures or unknown compounds.

Recent work using innovative custom-made impedance analyzers has revealed the capability of cellular platforms to sensitively detect extra-cellular stimuli. Novel EIS concepts comprising differential configurations and dedicated electrode set-ups and coatings in dual electro-optical cellular platforms with integrated microfluidics have been advanced. EIS assays on cellular sensors have targeted four main research directions:

- cells in suspension (eukaryotic cells (*151*) and red blood cells) with particular focus on cell cycle progression and evolution of cell shape as markers of cellular state.
- cells grown on (permeable) supports (mammalian cells MDCK, Caco2, and A6) to characterize cell adherence, proliferation and cell-cell communication in response to extracellular factors (e.g. Cd ions) and of altered lipid environment effect on cell physiology.
- microscopic modeling of clusters of interconnected cells and of nonspherical cell suspensions.
- development of differential EIS instrumentation (*177*). This approach has been applied for sensitive antigen-antibody detection (*164*) and recently for adherent whole cells (*178*).

For monitoring purposes either the evolution of overall impedance parameters (|Z| & phase) at defined frequencies or evolutions of derived parameters (R/σ and C/ε) have been used, while nonlinear fitting routines provided on line access to characteristic microscopic parameters. Cell cultures of adherent, polarizable cells (such as A6 or MDCK cells) are particularly suitable for electro-optical assessment of environmental effects (including nanoparticles), as cell growth is attachment dependent, cell polarization provides selective cell exposure (similar to whole organisms) and the specialized junctions (e.g. “gap”) between cells are sensitive indicators of early perturbations in cellular state (*179*). As such, the electro-optical assessment of adherent mammalian cells provides a potentially finer analytical platform to complement experiments on zebrafish embryos. When chemical stressors (e.g. $ZnCl_2$) are used, cell detachment from the surface can be monitored using both impedance modulus and phase, until complete sensor uncoverage. This effect is particularly relevant since this is the first step towards cell death. We use cells with attachment dependence at lower concentrations than cytotoxic (acute) ones. Figure 2 shows the evolutions of monolayer resistance and capacitance, at low frequencies, upon a toxic insult. In Figure 3 a similar test with adherent cells, reveals the evolution of the specific impedance of the cells subjected to various concentrations of a pore forming compound.

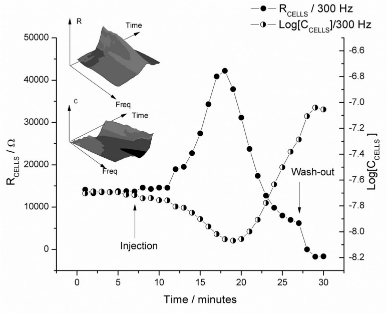

Figure 2. Time evolution of the Resistance/Capacitance of the cell monolayer upon Ethanol (45% final concentration) exposure. Inset 3 D evolutions of R and C as function of frequency and time.

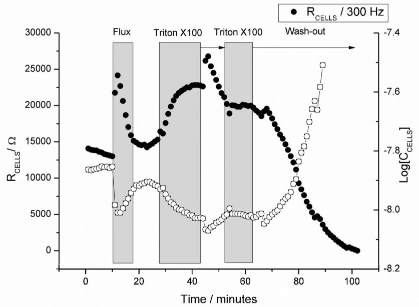

Figure 3. Time evolution of the Resistance/Capacitance of the cell monolayer upon Triton X 100 exposure at 0.01% and 0.02% final concentrations.

The results have been recorded using a custom made differential EIS system at the International Center of Biodynamics, Bucharest, Romania against the impedance of bare electrodes, as control. EIS is a non-invasive technique (tiny relative perturbations of the potential across cell membrane are induced by an applied electric field) and a “mature” field considering the availability of both appropriate instrumentation and, to some extent, of theoretical models (*152*, *180*) and data analysis approaches. This allows further refinements into *microscopic and structural data* of the measured frequency-dependent relationship between impedance (Z), conductivity (σ) and relative permittivity (ε_r) to provide cellular (cell membrane and cytoplasm) and morphological parameters (including cell shape and cell-cell connections).

As such, wide frequency range analysis advances over commercially available instruments (e.g. Acea Biosciences, US) where dimensionless parameters such as Cell Index (CI) are derived as a relative change in measured electrical impedance to represent cell status. Electro-Optical Investigation of pore forming compounds on cellular platforms reveals sensitivity to pore formation in the membrane as well as morphological changes and cell detachment due to low concentration of antimicrobial peptides and detergents (*181*, *182*).

These studies contribute to a deeper insight into the complex dynamics that occur in cell membranes due to cell interaction with extra-cellular compounds and support the feasibility of complementary electro-optical studies of engineered nanoparticles on cellular platforms. Our preliminary data in congruence with literature reveal that early changes in cell attachment, membrane properties and cell to cell communication are important indicators for toxicity assessment. Moreover, based on time lapse simultaneous electro-optical assessment of cell attachment on nanostructured surfaces, it has been shown (*178*) that morphological and electrophysiological parameters of cells can be predictably altered/engineered by nanoscale modulation of the chemical, physical, and topographical features of culture substrates highlighting possible bioeffects of adsorbed nanoparticles not only of the ones in suspension.

4.3.2. Surface Plasmon Resonance Assay

Among the optical detection methods for sensing living cells, surface plasmon resonance (SPR) is one of the most promising candidates for non-invasive detection assays because it does not require labeling agents and is amenable to high throughput analysis. Recent studies have demonstrated that SPR can be combined with living cells to monitor the effects of different molecular stimuli on cellular activity (*183*). Among the applications with potential in unraveling nanoparticle-cell interaction are studies of protein-protein, lipid-protein and ligand-protein membrane interactions (*184*) and immunophenotyping (*185*) peptide microarray for monitoring protein kinase activities in cell lysates developed based on SPR imaging (*186*). When applied on lipid bilayers as synthetic mimics of cell membranes, SPR measurements allow real-time assessment of the attachment, pore formation and membrane dissolution cascades involved in lythic peptide – membrane interactions (*181*, *182*). It is fully integrable with other techniques such as electrochemistry, optical and scanning probe microscopy.

4.3.3. TIRFM To Study Cellular Uptake and Assess Cell Membrane Dynamics

Optical sensing methods, such as nanoprobes and fluorescence microscopy are being extensively applied to monitor living cell activity, including mitochondrial membrane potential, Na and K dynamics. Total internal reflection fluorescence microscopy (TIRFM) (*187*, *188*) has been used, together with other microscopy techniques such as scanning confocal and near-field optical microscopy (SNOM) and dark field, for advanced optical assessment of nanoparticles within cells and interfacial reactions (*189*). In TIRFM when light is shone at an interface at angles above the critical one where total refraction occurs, an evanescent wave is formed in the lower refractive index medium, where the intensity drops exponentially with the distance from the interface. Thus, fluorescent molecules more than approximately 200 nm from the glass will not be excited, leading to a dramatic decrease in background fluorescence. For this reason, TIRF microscopy is the preferred single-molecule fluorescence imaging approach for studying plasma membrane-associated processes. Depending on the size and position within the cell, quenching or enhancing effects can be modulated for "in cell" imaging purposes (*190*).

4.3.4. Atomic Force Microscopy (AFM) To Study Cells – Nanoparticle Interaction

The study of adhesive and elastic (cell stiffness) properties of microbial and mammalian cells using AFM is proving to be an accurate tool for the real-time observation of the effects of metal ions and nanoparticles on cell physiology in their natural environment (*191*). Changes in cell stiffness were associated with reactive oxygen species generation (*192*) while cell response to nanotopographies of varying nanoparticle densities (*193*) reveal altered cell spreading actin network organization. In addition, AFM provides a sensitive method of measuring the force of nanoparticle–cell membrane interactions and the extent of cellular uptake of nanoparticles. Greater force of adhesion with the cell membrane, rapid internalization and specific intracellular trafficking as revealed by AFM can all substantiate our understanding of the interaction of nanoparticles on cellular systems (*194*). AFM has been used to investigate the effect of nanoparticles size and roughness at membrane level (*195*). Advanced AFM instrumentation for cell assessment in liquids, fully integrable with optical assays and with advanced electrochemistry options (such as NanoWizard II JPK Instruments, Germany) is commercially available. Interesting improvements pertain to hollow tips (*196*) for local liquid dispensing and stimulation of single living cells under physiological conditions and on line monitoring approaches of single cell mass growth (*197*) provide novel investigation tools of nanoparticle effect on cell dynamics. Nanoparticles with alternating striations of hydrophobic and hydrophilic ligands cross the cell membrane by a direct mechanism — a route that delivers them to the main compartment of the cell while leaving the membrane undisrupted (*198*). This alternative route has been recently speculated to create AFM tips that can have better compatibility with membranes allowing for long time monitoring of cell structures (*199*).

The majority of the micro-analytical systems are developed for the extraction and analysis of intracellular contents for metabolites and genetic material. Relatively few systems have been reported for the detection of secreted biochemical molecules by live cells: albumin production and p-nitrophenol to measure alkaline phosphatase activity in osteoblasts (*200*) and amperometric detection with a carbon fiber microelectrode (*201*), micro-electrophoresis based immunoassay (*202*) and on-chip ELISA systems (*203*). In a recent approach (*204*), microelectrodes were used to quantify extracellular ROS generation in response to a toxic insult.

5. Zebrafish Embryos As a Model Toxicological Target

Traditionally, toxicity testing has focused on mammalian models with particular emphasis on rodents due to obvious similarities in physiology and organ systems. While use of these systems provides extensive information about the levels and nature of toxicity of many compounds, there are also drawbacks to using these systems. Toxicity testing in mammals requires a large number of animals with demanding housing requirements and time-consuming exposure protocols. Exposures require relatively large amounts of the test substance to produce effects and analysis of toxicity are often lengthy and complicated. Also, testing on early stages presents additional problems due to inaccessibility during in utero development. As a result, mammalian animal trials are typically expensive. There are also increasing demands from the public to limit the number of animals used in toxicity trials. While trials on mammalian subjects are still critical for determination of toxicity, other animal systems are being developed which are more accessible and less costly to house and test. These other systems can then be used for identifying toxic substances and be highly predictive for what compounds to proceed with in mammalian tests.

One such model is the zebrafish system. This system circumvents a number of the previously mentioned disadvantages in mammalian systems. Zebrafish are a common pet store variety of fish but in recent years, there has been an increasing use of the zebrafish system in studies related to both early development as well as later events of organogenesis. Not only have zebrafish embryos been increasingly used in developmental studies but the embryos have been also increasingly used in both toxicological and drug discovery investigations.

5.1. Why Are Zebrafish Useful for Whole Animal Toxicology Investigations?

The zebrafish system is amenable to the whole animal research that toxicology testing requires and there has also been an enormous effort on a number of different fronts to create a group of tools with which to analyze zebrafish both molecularly and phenotypically. Zebrafish are small vertebrates in which adults grow to about four to six centimeters in length. Adults are able to be housed in relatively high density with about 40-50 animals in a ten-liter tank and are able to breed year round. A single pair of adults can produce anywhere from 100 to 500 externally developing embryos in a single mating. This means that adults can reliably and repeatably produce embryos for toxicological testing.

Zebrafish embryos are also well suited for toxicology testing. Embryogenesis is only five days going from a single cell to an organism able to move and feed independently. Embryos are optically transparent making much of development and organogenesis visible in the living embryo (*205*). In addition, transgenics expressing green fluorescent protein (GFP) under the control of specific promoters label different cells and structures, which can also be viewed in the living embryo under fluorescent light. Embryos can also be grown without pigment, which allows for visualization of embryogenesis and easy detection of developmental defects. In addition to the ease of embryo growth and observation, there has been extensive development of zebrafish as a genetic system. The zebrafish genome has been sequenced and is in the process of being annotated (*206*). There are also techniques to manipulate the genome as well as gene expression (*207*). As a result, genetic effects due to nanoparticles toxicity can be pursued.

Embryos are grown in multi-well plates with as little as 500 ml of liquid due to their small size of less than 1 millimeter. This allows for the use of drastically smaller amounts of compounds used in testing. The drugs are also able to be added directly to the embryo water as they diffuse into the embryos without the need for injection or elaborate exposure protocols. These characteristics make toxicology testing in zebrafish practical for larger scale operations.

There are also a host of other tools that have been under development to further analyze specific molecular pathways that become affected with the addition of toxic substances. These tools will allow for comprehensive investigation of what organs and cell types are affected during exposure. Changes in gene and protein expression in the whole embryo can be analyzed by RNA in situ hybridization and immunohistochemistry, respectively (*207*). The zebrafish genome has been sequenced with a 6.5 to 7 fold coverage with substantial annotation (Wellcome Trust Sanger Institute *Danio rerio* sequencing project online http://www.sanger.ac.uk/Projects/D_rerio/) allowing for the rapid identification of gene structure. Rapid "knock-down" of protein expression in the live embryo can be obtained by injection of anti-sense oligos (morpholinos) to a specific gene (*208*) to determine if the gene plays a role in the toxic response. Expression profiling after exposure can be done with microarrays using RNA from whole embryos or from dissected organs. Also, transgenic zebrafish are now easier and faster to create using Gateway cloning vectors (*209*, *210*). Alteration of gene expression can block or change the toxic response demonstrating that the gene plays a role in the process.

5.2. Why Use Zebrafish for Toxicology?

At first glance, zebrafish do not seem as though they would have much relevance to human health. However, upon further investigation, zebrafish have strong similarities to mammals in the way that they respond to toxic substances. In a study to determine whether zebrafish embryos respond in a similar manner to mammals, 18 drugs of various types were applied to determine the LC50. The LC50 of these drugs were found to be comparable to mammals (*211*). In another study, an assay was developed to screen zebrafish embryos for their ability to detect the teratogenic potential of chemicals (*212*). This assay used a number of organ systems sensitive to toxic chemicals. The level of affect in the organ system was then used to assign a concentration at which there is no-observed-adverse-effect (NOAEL). The LC25 for each substance was determined and a ratio of the two numbers was generated. This ratio enabled the correct classification of 87% of the 31 chemicals tested as a teratogen. Both of these studies suggest that zebrafish is an effective predictor of compounds that are toxic in mammalian systems. In recent years, zebrafish have been developed as a model system to further understand many areas of early pattern formation (*213*), organ development (*214–216*), metabolism (*217*), and toxicity response (*218*, *219*).

While zebrafish embryos have been shown to be extremely useful in predicting both the types of chemicals and the concentrations at which they will be toxic in mammalian systems, these exposures have also demonstrated similar organ and behavioral defects to mammals. Therefore, not only are zebrafish useful for identifying toxic chemicals but they are also a useful tool for identification of the mechanism of action. As a result, all of the tools available for zebrafish can be used to define a mechanism of action for toxicity.

One group of cells that are sensitive to toxicants is the hair cells of the inner ear. The same aminoglycoside antibiotics that have been shown to affect hair cells of the human ear (*220*) also affect the hair cells of the zebrafish inner ear as well as the lateral line (*221*, *222*). The lateral line are comprised of individual organs composed of hair structures similar to hair cells in the ear along the length of the body used to detect changes in water current making them critical for locomotion and feeding. The similarity of the lateral line to inner ear hair cells has subsequently allowed zebrafish embryos to be used to screen for the ototoxicity of other drugs as well as otoprotecive agents (*223*, *224*). In addition to drug exposure, metals such as lead, mercury, manganese, platinum, tin, and cadmium have also been demonstrated to cause hearing loss in mammals (*225*). In zebrafish, cadmium exposure has been demonstrated to result in cell death of olfactory cells (*226*).

Another organ that is sensitive to drugs is the heart in the form of life-threatening arrhythmias due to defects in cardiac repolarization (*227*, *228*). Several pharmaceuticals have been taken off the US market due to these problems. Drugs that have been demonstrated to cause these arrhythmias in humans also cause the same defects in the zebrafish embryo. In a recent study, drugs that cause QT prolongation in humans cause a similar defect of bradycardia in zebrafish (*229*) demonstrating similarities in molecular pathways and physiology. The response of both hair cell defects and cardiotoxicology in zebrafish to drugs and environmental pollutants that cause the same type of defects in humans and other mammals demonstrates the utility of zebrafish for not only predicting what substances will be toxic, but the system will also be able to identify the specific organs and pathways that are affected across many vertebrates.

In a recent review by Sukardi et al. (*230*), the authors discuss the use of omics approaches for analysis of transcriptome, proteome and metabolome. Zebrafish embryos could then be used to generate a specific omic signature for a variety of chemicals. These omic signatures could then be used to identify unknowns from environmental samples. Alternatively, changes in these signatures could be used to determine if subsequent drug applications or genetic manipulations block or ameliorate the initial toxicity.

5.3. Nanotoxicity in Zebrafish Embryos

With the similarities of the zebrafish to mammals, this system provides an excellent opportunity to determine levels of nanoparticle toxicity to both aquatic systems as well as their impact on human health. As discussed previously, nanoparticles are already in high use for a variety of applications including sensing, catalysis, environmental remediation, medical diagnostics, imaging and drug delivery (*231*, *232*). However, their interactions with biological systems and levels of accumulation within individual organisms are largely unknown (*231*, *233*, *234*).

To determine levels of nanoparticle toxicity, investigators have taken the same approach as with addition of drugs to the zebrafish system by adding them directly to the embryo water. After exposure in the aqueous growth medium, obvious incorporation of the particles are visible within the pharynx and intestine of the embryo (Figure 4) (*51*). Application of a variety of metallic nanoparticles to zebrafish embryos has demonstrated that toxicity is dependent on the type of metal. Depending on the type of metal, the nanoparticles can result in widely differing LC50s. In a study by Griffitt et al., silver and copper nanoparticles had the highest toxicity (in the 7.2 and 0.71 mg/L range, respectively) while other nanoparticles such as aluminum, cobalt, nickel and titanium had toxicities over 10 mg/L (*235*). Aqueous suspensions of nanoparticles release ions over time but dissolution is not soley responsible for the observed toxicity. When a similar concentration of respective ions are added to zebrafish embryos, they do not result in the level of toxicity observed when nanoparticles are present (*1*, *235–237*). In addition to mortality with determination of LC50, organ defects (intestinal epithelial cells, cartilages and muscles) created during nanoparticle exposure can also be investigated with zebrafish embryos. Histological analysis of exposed embryos reveals aggregated particles primarily within the pharynx and anterior intestine.

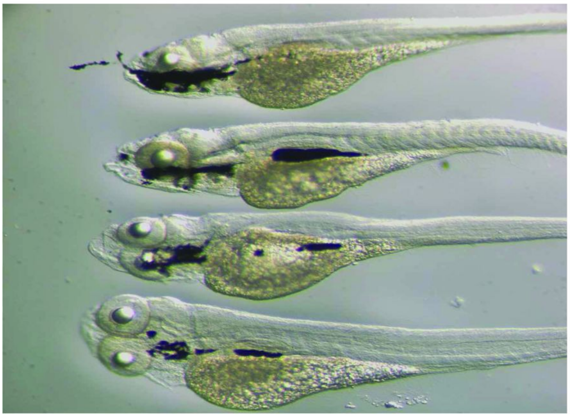

Figure 4. Incorporation of nanoparticles in embryos (dark spots are aggregated nanoparticles), from reference (1).

Furthermore, we have demonstrated that there are differences between the defects caused by nickel nanoparticles and defects related to soluble nickel. Nickel nanoparticles result in intestinal defects but even at much higher concentrations of soluble nickel, there are no observable problems in the intestine (*1*). This suggests that nanoparticles cause toxicity by different routes than their corresponding metal. Other than dissolution of nanoparticles, another method by which nanoparticles may increase toxicity is by causing a rise in production of ROS (*238*).

While different composition nanoparticles play a large role in toxicity, different size nanoparticles of the same metal do not appear to generate much difference in toxicity. We have found that exposure of zebrafish embryos to different size nickel nanoparticles results in similar LC50s for particles of 30, 60 and 100 nM (*1*). Another study with silver and gold nanoparticles of different sizes also does not result in widely different toxicities (*238*).

Differences in shapes, however, can result in greater differences in toxicity. We have found that dendritic nickel particles with groups of 60 nm entities have a greater toxicity than any of the spherical nanoparticles or soluble nickel (*1*). Once nanoparticles enter the embryo, differences in shape may affect retention time and total levels of accumulation. Future experiments will need to investigate sublethal affects on target organs in order to tease out more information as to how nanoparticles are initiating the toxic affects. Also, alteration of nanoparticle surface properties is likely to make nanoparticles more biocompatible.

6. Sensors As Predictive Tools for Nanotechnology Risk Assessment

While conventional *in vivo* and *in vitro* methodologies for nanotoxicity assessment have provided valuable information on the effects of nanoparticles, many of these methods are viability assays that do not necessarily predict *in vivo* responses and do not provide an understanding of the basic interaction and specific molecular effects of these nanostructures with biological systems. Many questions related to the origin of the nanotoxic response, the site of molecular and biochemical action (e.g. inflammation, release of ROS), the effect of surface reactivity, surface charge or nanoparticle composition are still unanswered. To gain deeper understanding of nanoparticle-induced toxicity and develop a truly predictive model for nanotechnology risk assessment, alternative analytical methodologies are needed to unravel the mechanism of nanotoxicity and predict *in vivo* response of nanotechnology products.

Several types of electrochemical sensors that have been used in the past to perform real-time measurements in cell cultures and *in vivo* conditions are potentially very attractive tools for nanotoxicity assessment. Such sensors are small (micron to submicron dimensions) and easily implantable and can provide real-time assessment of the changes in the concentration of cellular messengers and biochemical markers *in vitro* and *in vivo*. Electrochemical sensors can be used in the nanotoxicology field to study biochemical and cellular events involved in the cytotoxic response during/after nanoparticle exposure. For example, electrochemical microsensors can offer real-time continuous monitoring of ROS and inflammation markers (NO, melatonin) as well as quantification of release of soluble ions secreted into the extracellular space with minimal perturbation of the cellular and *in vivo* model (*239*). Few studies of electrochemical assessment of the interaction of nanoparticles with cells have been reported (*240*, *241*). These studies have been performed on cellular systems. Their use for assessing *in vivo* toxicity has not been reported. Specific examples of such studies and a discussion of the potential of the sensing technology in nanotoxicology are discussed below.

6.1. Electrochemical Sensors for Assessing Cellular Messengers and the Redox Status of Cells

Carbon fiber microelectrodes have been used to monitor dynamic changes in the secretion of chemical messengers released from model cells (murine peritoneal mast cells) exposed to nanoparticles (*154*, *241*). The potential of this technology to probe nanoparticles-cell interactions was demonstrated with cells exposed for 48 hours to Au nanoparticles of 28 nm diameter by measuring the serotonin exocytosis from cells. Single cell amperometry experiments revealed significant trends in cell function including the dynamic of serotonin released, which occurred faster in the presence of the nanoparticles as compared to unexposed cells and the kinetic of these processes. This work demonstrates the ability of this method to provide dynamic real-time detection of chemical messengers at single cell level and its potential in studying nanoparticle-cell interaction during nanoparticle exposure (*154*, *241*, *242*). Though this research focused only on serotonin, other chemical messengers can also be detected using electrochemical sensors. Examples include NO, melatonin, cytokines and reactive oxygen species, as described below.

The propensity of nanoparticles to induce oxidative stress as a major mechanism of nanoparticle effects is well established (*132*, *140*). Depending on the type of material and experimental conditions, nanoparticles can either induce ROS production or scavenge free radicals (*243*). Recent studies focus on ROS production as a significant marker of toxicity for a wide variety of nanoparticles (*234*, *244*, *245*). This mechanism has been proposed to be an important pathway for toxicity for a variety of nanoparticles including nano–C60, III–V and II–VI quantum dots (QD) and titania. There has been some debate in the literature regarding the origin of ROS production, particularly whether the ROS were generated by the nanoparticles themselves (see for instance cell free experiments demonstrating the ability of nanoparticles to generate ROS (*246*, *247*)) or by cellular activity physically disrupted by the nanoparticle. Moreover, studies have demonstrated that nanoparticles can induce glutathione depletion indicative of oxidative stress in various cell lines (*248*, *249*). This can induce specific signaling pathways as well as pro-inflammatory gene expression (*250*). Therefore, the evaluation of the oxidative potential of nanoparticles is an important parameter in assessing nanotoxicity. The amount of released superoxide depends on the presence of superoxide-scavenging enzymes, the age of the cells, the exposure route, the amount of nanoparticles, the integrity of cell junctions and the temperature. Clear identification of the source of ROS release (mitochondrial and/or NAD(P)H) requires careful choice of specific inhibitors, since phorbol 12-myristate 13-acetate, a compound known to activate NAD(P)H oxidase, is able to elicit similar dynamics as nanoparticle exposure. Moreover, nanoparticles can damage DNA (*116*); hence the identification of the mechanism involved is of major importance.

An electrochemical cytochrome c-based superoxide biosensor was constructed (*204*) to evaluate the extracellular production of ROS species. This simple, miniaturable superoxide biosensor has minimal interferences from H_2O_2, ascorbic acid, and uric acid, and appropriate sensitivity to small amounts of superoxide produced by cells (*251*). The sensor was tested with calcium oxalate crystals (*204*) in the extracellular space upon nanoparticle exposure/accumulation on renal cells as model systems. ROS release proved to be a temporary, non-monotonous process, suggesting that only a continuous monitoring, as enabled by electrochemical extracellular sensors, can evaluate correctly the extent of the oxidative stress at cell level, while end point detection failed to do so.

In addition to electrochemical sensors, an optical immunosensor was developed to monitor cellular immune response to stress induced by the presence of nanoparticles for assessing nanotoxicity *in vitro* (*252*). The optical label free immunosensor was able to determine inflammatory cytokines (e.g. Interleukin 8) in cell culture medium as an indication of inflammatory response, and differentiate between nanoparticle exposed and unexposed cells.

6.2. Electrochemical Microsensors for *in Vivo* Assessment of Nanotoxic Response

In addition to *in vitro* systems, electrochemical microsensors can also be used to determine nanotoxic responses and chemical messengers *in vivo*. For example, in zebrafish embryos use of electrochemical sensors can provide real time signatures of exposure to toxic substances. Exposure to toxic substances should produce a specific set of electrochemically active compounds that may be able to predict the type of compound to which the embryos are exposed and similar to omic signatures, changes in electrochemically active compounds could also reveal positive or negative changes in exposure due to subsequent manipulations. Increases in reactive oxygen species at the site of exposure will likely be one alteration. Additionally, toxic compounds are likely to be inhaled or ingested causing accumulation and extended exposure times depending on the substance. Within the digestive system, there are cells with sensors facing the lumen that respond to noxious substances and produce serotonin to signal changes in smooth muscle and enteric neurons, which then alter motility of the intestine (*253*). We have previously used differential pulse voltammetry with implanted carbon fiber microelectrodes to measure changes in the intestinal level of serotonin of live embryos (*254*). Levels of serotonin, other neurotransmitters, and reactive oxygen species determined by electrochemical sensors, should provide a different real time signature for a variety of toxic substances. The microelectrodes were implanted at various locations in the intestine of intact embryos. Serotonin levels of 29.9 nM were measured *in vivo* in normal physiological conditions. Measurements were performed in live embryos without additional perturbation beyond electrode insertion. The sensor was able to quantify pharmacological alterations in serotonin release and identify different levels along the anterior/posterior axis with high spatial resolution. This type of sensor can be extremely useful to monitor neurological damage at the nanoparticles accumulation site. Serotonin, melatonin and NO are markers of inflammation and ROS release is believed to occur during nanoparticles exposure (*43*, *44*, *48*), (*73–75*). In the future, such sensors can be used to measure localized *in vivo* NO and ROS release at the nanoparticles accumulation site in intact embryos.

7. Conclusions

Progress in nanotechnology has facilitated development of a myriad of nanotechnology products and devices that have already had a major impact in many fields. However, before the full potential of the nanomaterials can be realized, there must be extensive and systematic studies of the toxicity of these particles. Although a great deal has already been learned about these nanoparticles, studies of the molecular events involved in the cytotoxic response in biological systems, bioaccumulation and toxicology are only in their infancy. Both fundamental studies at molecular levels and development of analytical methodologies for assessing toxicity are needed in future research activities. Research is needed to identify key factors that can be used to predict toxicity, permit targeted screening and allow designing materials with controlled toxicological impact. Monitoring cell behavior under varying conditions and understanding molecular interactions in the context of a living cell is expected to have a considerable impact on nanotoxicity risk assessment. Particular emphasis should be given to monitoring changes in cell membrane properties in response to low concentrations of nanoparticles (the membrane acts as a primary filter and regulator in cell signaling) and the way that nanoparticles penetrate cellular membranes. On the other hand, *in vivo* measurements of nanoparticles toxicity on intact animals are still valuable for nanotoxicity assessment. The use of *in vivo* whole animal work and *in vitro* cellular assays in conjunction with electrochemical microelectrodes and specific biophysical methods will bring a new wave of methodologies with potential to provide additional, complementary toxicity information to commonly used viability assays. This will allow localized study of nanotoxic effects at particular sites in a dynamic real-time mode. These efforts will also contribute to establishing standards and safety regulations, e.g. maximum levels related not only to nanoparticle chemical composition but also to their respective structural properties. Novel methodologies could also facilitate development of models of nanoparticle toxicity for predicting their potential impact on health and the environment.

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